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SOME ASPECTS OF ION UPTAKE BY MARINE ALGAE

The Uptake of Radioactive Phosphate by *Laminaria digitata*

by

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CONTENTS

Abbreviations

ATP	Adenosine triphosphate
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
G-1-P	Glucose-1-phosphate
G-6-P	Glucose-6-phosphate
F-6-P	Fructose-6-phosphate
F-D-P	Fructose-1,6-diphosphate
3PGA	3-phosphoglyceric acid
23PGA	2,3-diphosphoglyceric acid
M-6-P	Mannose-6-phosphate
M-1-P	Mannitol-1-phosphate
NAD	Nicotinamide adenine dinucleotide
$NADH_2$	Dihydronicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
$NADPH_2$	Dihydronicotinamide adenine dinucleotide phosphate
PM	Metaphosphate
PP	Pyrophosphate
PO	Orthophosphate
$P^{32}O$	Radioactive orthophosphate
R-5-P	Ribose-5-phosphate
UDP	Uridine diphosphate
UTP	Uridine triphosphate

Reference Compounds

The following reference compounds were used throughout the work:

Compound	Salt	Source
ATP	disodium salt	Sigma (London) Chemical Company Ltd.
ADP	sodium salt	
AMP	sodium salt (equine muscle)	
G-1-P	disodium salt	
G-6-P	disodium salt	
NAD	disodium salt (yeast)	
$NADH_2$	disodium salt	
NADP	sodium salt	
$NADPH_2$	tetrasodium salt	
UDP	sodium salt	
UTP	sodium salt	
F-6-P	barium salt	The British Drug Houses Ltd., Poole, Dorset.
FDP	tetrasodium salt	
3 PGA	barium salt	
23 PGA	barium salt	
M-6-P	barium salt	
R-5-P	barium salt	
PP	tetrasodium pyrophosphate	
PM	sodium metaphosphate	

Mannitol-1-phosphate. This reference standard was prepared by the reduction of M-6-P with potassium borohydride (Wolff and Kaplan, 1956).

INTRODUCTION

The Importance of Phosphates in Ion Accumulation

The phosphate bonds of biologically interesting substances are usually classified as high- and low-energy bonds on the basis of the free energy change occurring on hydrolysis. However, as it is often difficult to discriminate between the two types of bond, the proposal by Klotz (1957) to indicate the "energy wealth" of phosphate bonds as the phosphoryl transfer potential (Ptp) is more acceptable. The value of Ptp, expressed in kcal., corresponds to the maximum amount of biochemical work that can be obtained from a given phosphate bond.

Utilization of the Ptp stored in ATP systems follows a substantially common pattern in all organisms and involves the action(by kinases or phosphate transferases) upon substrates, catalysis of synthetic reactions by phosphorylases or pyrophosphorylases, and finally regeneration of inorganic phosphate by phosphatases. The dependence of such a general scheme on Ptp energy has been demonstrated at the molecular level and observations *in vivo* suggest strongly the involvement of phosphorylation in a number of physiological processes, e.g. growth, control of rate of respiration, photosynthesis and active uptake of solutes.

The relationship between respiration and ion absorption and the problem of whether active ion uptake depends directly on electron transfer or Ptp generated by oxidative reactions has been studied by Laties (1959a), Sutcliffe (1959) and Robertson (1960).

The main evidence for the requirement of Ptp for ion uptake is based on its inhibition by uncoupling agents such as 2,4-DNP. Robertson (1960) stated that the DNP effect could depend on the fact that electron transfer

is effective for active ion uptake only when coupled to phosphorylation, even if ATP synthesis is not the determining factor. This is difficult to test and remains hypothetical. Recent evidence indicates that ion uptake is inhibited not only by uncoupling agents but also by inhibitors, such as cyanide and carbon monoxide, which do not change the pathway of electron transfer, and that inhibition occurs even in cases where the overall oxygen uptake is not affected. Hackett et al. showed the inhibition of inorganic phosphate uptake by KCN in aged potato discs whose respiration is largely KCN insensitive.

On the other hand, not only ion uptake but also the uptake of non ionized compounds, such as sugars, is inhibited by pure uncouplers and by inhibitors of cytochrome oxidase. Pennell and Weatherley (1958) showed that KCN, azide, DNP and anaerobiosis inhibit penetration of sugars into leaf discs.

The above evidence, together with the difficulty of establishing a strict quantitative correlation between solute uptake and solute dependent oxygen uptake (Laties, 1959a), strongly suggests that some common basic Ptp energy-utilizing mechanism is necessary for active uptake of solutes of any kind. This does not contradict evidence in favour of existence of specific carriers or mechanisms for the uptake of the different molecular species, as the synthesis and functioning of these carriers or mechanisms could depend on Ptp. In connection with this, the results of Laties (1959b) may be quoted. Laties found that in aging potato discs chloride uptake rapidly increases, the increase being reversibly inhibited either by low temperature of by 2,4-DNP. Since both conditions induce a lowering of Ptp in the cells, these results could be interpreted by the assumption of a Ptp energy-dependent synthesis or activation of a carrier.

Phosphates found in Plants

(a) Sugar phosphates

Sugar phosphates are very difficult to extract from plant tissue because of their low concentrations and the presence of very active plant phosphatases. The metabolic state of the plant may determine the concentrations of these intermediates. Since the concentrations are low and the amount of material passing through these metabolic pools in a short time is great, there may be profound variations resulting from external changes prior to analysis. The value of results for compounds of such transient nature can only be realised by further understanding of their metabolism.

Analytical methods for sugar phosphates in animal and isolated enzyme systems are well known. Leloir (1951) gave a review of the isolation, structure, properties and analysis of sugar phosphates. The occurrence and metabolism of phosphorylated compounds in plants were reviewed by Albaum (1952). Identifications of photosynthetic intermediates of carbon dioxide reduction were summarized by Buchanan et al. (1952) and their transformations were discussed by Bassham et al. (1954). As yet there is no comparative biochemical study of the distribution of phosphate esters in plants. However, a preliminary survey by Norris & Calvin (1954) covers the plant kingdom quite well, but much investigation of the identity and concentrations of phosphates remains to be done.

Early attempts to isolate phosphorylated compounds from tissue used precipitation techniques involving barium ions. The precipitation techniques, whilst being successful with animal tissues and microorganisms, were not wholly successful when applied to plants. The first attempt,

carried out by Albaum & Umbreit (1943) on oat seedlings, led to the certain identification of only inorganic phosphate, fructose-6-phosphate, hexose diphosphate, phytic acid, and labile phosphorus which might be associated with ATP. Emerson et al. (1944) had the same type of difficulties with attempts to fractionate the phosphorus-containing substances in Chlorella identifying with certainty only inorganic phosphorus and hexose diphosphate. Working with Euglena, Albaum et al. (1950) demonstrated the presence of inorganic orthophosphate, pyrophosphate and metaphosphate, adenylic acid, ATP, ADP, DPN, glucose-1-phosphate, fructose-6-phosphate, hexose diphosphate, phosphoglyceric acid and riboflavin phosphate.

The emergence of chromatography provided a very useful tool in the analysis of plant extracts. Albaum & Scher (1952) demonstrated the presence of the following compounds in mung beans, using a combination of chromatography and autoradiography: glucose-1-phosphate, glucose-6-phosphate, fructose-6-phosphate, inorganic orthophosphate, phosphoglyceric acid, phytic acid and phosphopyruvic acid.

(b) Phosphorus containing compounds in glycolysis

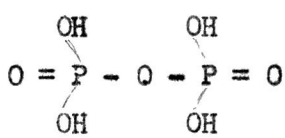
There is now a wealth of evidence to show that phosphorylated compounds in plants play much the same role in glycolysis as they do in animal tissue and in microorganisms. Tewfik and Stumpf (1949) demonstrated that extracts of many plant tissues can enzymatically split hexosediphosphate into phosphoglyceraldehyde and dihydroxyacetone phosphate and a review article by Stumpf (1952) presented evidence for the existence of a complete glycolytic system from consideration of enzymes activities. Albaum, Ogur & Hirschfield (1950) showed that ATP did occur

in plants and the involvement of this substance, both alone and in association with pyridine nucleotides in the phosphorylation reactions of photosynthesis, has been adequately confirmed particularly by the work of Arnon (1961) and co-workers.

(c) Inorganic condensed phosphates in plants

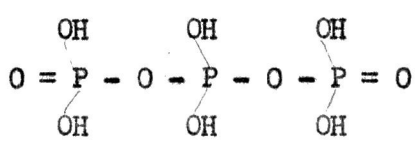
The condensed inorganic phosphates include substances over a wide range of polymerization and those possessing both low and high degrees of polymerization are of biological interest. The lower members of the group, i.e. up to members of 4 phosphoryl groups per molecule, can be classified into two series which differ according to the degree of hydration: those of the first series are open chain polyphosphates and related to pyrophosphate. Members of the second series contain one molecule of water less than the corresponding member of the first series, e.g.

Pyrophosphoric acid

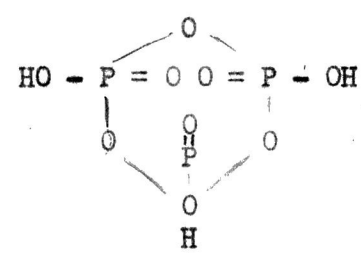


Dimetaphosphoric acid?

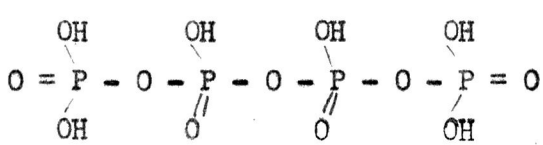
Tripolyphosphoric acid



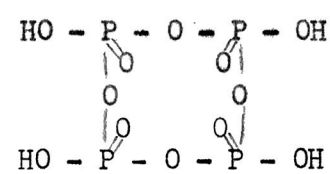
Trimetaphosphoric acid



Tetraphosphoric acid



Tetrametaphosphoric acid



The general formula of compounds of the second series is $(MPO_3)_n$ where M signifies a univalent cation. It has been shown by Hull (1941) by means of radioactive phosphate that no exchange occurs in solutions between ortho-, pyro-, and metaphosphates.

Most naturally occurring condensed phosphates are members of the first series, i.e. open chain polyphosphates. The majority of naturally occurring polyphosphates, which can be detected by chemical methods, exist in either of two forms: one easily extractable with cold TCA, the other insoluble with cold TCA, but soluble in hot TCA, perchloric acid or dilute alkaline or neutral salt solutions. The two fractions differ in their physiological behaviour, the TCA insoluble group being reported as more actively involved in metabolism (Wiame, 1949). Both forms are easily hydrolyzed within 7 minutes by 1 N HCl at 100°C and are thus characterised as "7-minute phosphates".

The first reported isolation of metaphosphate from plants was by Liebermann (1888, 1890) who extracted the substance from yeast. Many years later, MacFarland (1936) confirmed the presence of metaphosphate in yeast. Houlahan & Mitchell (1948) found inorganic polyphosphate as a normal cell constituent of wild Neurospora strains. Certain Neurospora mutants accumulated up to tenfold the normal concentration of polyphosphate. Sommer & Booth (1938) found both pyrophosphate and metaphosphate in algae. When the cells were kept in phosphate free media, orthophosphate and pyrophosphate decreased at a greater rate than did metaphosphate. Hardin (1892) reported the occurrence of metaphosphate in cotton seeds.

Stich (1953, 1955) observed that the polyphosphate granules in Acetabularia mediterranea increased both in number and size in light, whereas they decreased in darkness. This suggested a connection between

polyphosphate synthesis and photosynthesis. In support of this, it was shown that after 24 hours exposure of the algae to $P^{32}O_4$ in the light P^{32} accumulated in the granules. In darkness or in light under the influence of 2,4 DNP the accumulation was reduced. Cyanide caused complete suppression of accumulation. The author assumed that polyphosphate synthesis can proceed only if photosynthesis and oxidative phosphorylation are unimpaired. The earliest thorough investigation of polyphosphate synthesis in algae was carried out by Wintermans (1955) who demonstrated that Chlorella vulgaris suspensions in the light converted orthophosphate into intracellular polyphosphate, the majority of which was TCA insoluble. He found that phosphate fixation could continue for several hours and was greater in the absence of CO_2 than in its presence. The reaction did not require oxygen; nitrate had no effect, but glucose was inhibitory. Polyphosphate formation in light was much less sensitive to phenylurethane than was photosynthesis, but both photosynthesis and polyphosphate synthesis were inhibited to the same degree by DNP, sodium azide and sodium fluoride. Wintermans conclusions were that the energy-rich phosphate groups, formed in the photochemical reaction of photosynthesis, can be stored as polyphosphate if the further reactions of photosynthesis are curtailed by lack of CO_2. In algae, polyphosphate tends to accumulate when the energy from the photochemical process of photosynthesis cannot be used for normal synthetic or other energy-requiring reactions. However, there is no direct evidence that algae cells are able to satisfy even part of their energy requirement by using polyphosphate as a source of stored energy.

By way of summary, Table I shows some phosphorylated compounds which have been discovered in plant tissue. Table I is by no means complete, but

is representative.

Table I

Compound	Where observed	Reference
Fructose-6-phosphate	oat seedlings Euglena mung bean	Albaum & Umbreit (1943) Albaum *et al.* (1950) Albaum & Scher (1952)
Glucose-1-phosphate	Euglena mung bean	Albaum *et al.* (1950) Albaum & Scher (1952)
Glucose-6-phosphate	mung bean potato tubers	Albaum & Scher (1952) Arreguin-Lozano & Bonner (1949)
Fructose-6-phosphate	oat seedlings Euglena	Albaum & Umbreit (1943) Albaum *et al.* (1950)
Phosphoglycerate	Euglena mung bean	Albaum *et al.* (1950) Albaum & Scher (1952)
Metaphosphate	Euglena Yeast Yeast Cotton seed	Albaum *et al.* (1950) Liebermann (1888, 1890) MacFarlane (1936) Hardin (1892)
Pyrophosphate	Euglena	Albaum *et al.* (1950)

(d) Level of phosphorylated intermediates in plants

Although the presence in plants of most of the phosphate compounds that occur in animal tissues and in microorganisms is well documented, relatively few data are available concerning the average concentration of the main phosphorylated constituents and their changes in relation to various physiological conditions.

Concentrations of some of the intermediates of photosynthesis and respiration were measured by Benson (1952) and Calvin & Massini (1952) using P^{32} and C^{14}. Benson grew Scenedesmus in P^{32} labelled nutrient and measured the radioactivity in sugar phosphates separated by paper chromatography. The specific radioactivity (counts per minute per gram atom P) was derived from a phosphorus analysis and radioactivity measurements

on a sample of the uniformly P^{32} labelled plant material. From the specific radioactivity, the known number of phosphorus atoms in the compounds and their radioactivity, the data quoted below were calculated:

Compound	P^{32} Analysis 4% CO_2, 20°C
Phosphoglycerate	5.7×10^{-3} M
Glucose monophosphate	1.1×10^{-3} M
Fructose monophosphate	2.7×10^{-4} M
Sedoheptulose monophosphate	1.0×10^{-4} M
Ribulose diphosphate	1.0×10^{-3} M

In a review article on phosphorylation in higher plants, Marré (1961) states that hexose monophosphates appear to predominate among sugar esters, their concentration ranging between 1 and 6 μM/gm fresh weight. Glucose-1-phosphate and fructose-1,6-diphosphate concentrations are significantly lower, ranging between 0.3 and 2 μM/gm fresh weight. According to data available, ATP and ADP concentrations vary around 0.2 to 1 μM/gm fresh weight.

The scant data available in this field make it very difficult to draw any general picture correlating changes in concentration of phosphorylated components with physiological conditions. The concentrations of these phosphates are known to depend on pH (Oullet & Benson, 1952), light intensity (Calvin & Massini, 1952) and the partial pressure of carbon dioxide (Wilson, 1954). The age of the organisms, temperature and cultural conditions also have an effect.

The concentrations of sugar phosphates in plants, then, are highly dependent upon their metabolic state and may change profoundly within a short time. Consequently, it is advisable that analysis for phosphates should be accompanied by a complete description of the condition of the

plant material and by the method of preparation of the sample.

Extraction of Phosphate Esters from Plant Tissues

Sugar phosphates are not normally encountered in plant analysis for two reasons. Their concentrations are very low, usually ten to a hundred times lower than those of sucrose or the free hexoses; and the very active phosphatases rapidly hydrolyze these phosphate esters unless the enzyme is quickly denatured during extraction. Benson (1955) pointed out that the phosphatases are active over a great temperature range, even in non-aqueous media and that the most active phosphatase activity remains attached to the plant tissue. These observations were made from a $C^{14}O_2$ photosynthetic experiment on barley seedling leaves. Extraction of phosphorylated substances, therefore, requires that the phosphatases be quickly and permanently destroyed and that cellular material be separated from the extract as soon as possible.

Cold dilute trichloracetic acid (TCA) is normally employed for the extraction of sugar phosphates. Rapid denaturation in boiling 80% methanol ensures that the phosphates are obtained in the concentrations existing at the instant of killing. Extraction in successive volumes of TCA ($0^{\circ}C$) is carried out until no further phosphorus is extracted. The rate of extraction depends on the degree of subdivision of the material. The rate of enzyme denaturation in such an extraction could be expected to be rapid compared with possibly changes in concentrations of intermediates during the extraction.

A further method of extraction of phosphate esters from plants entails the use of boiling 80% aqueous alcohol for one or two minutes. This is said to be sufficient to denature plant phosphatases. The major

fraction of sugar monophosphates along with free sugars, amino acids and plant acids are extracted by the time chlorophyll appears to have been extracted. Further boiling in 20% ethanol is required to remove phosphoglyceric acid, ribulose diphosphate and hexose diphosphates.

Separation of Sugar Phosphates Present in Plant Extracts

The early work on phosphorylated substances in plants was concerned with the measurement of phosphorus in TCA insoluble residue and in protein-free supernatant after filtration or centrifugation. The acid soluble fraction was assayed for inorganic phosphorus and organic phosphorus, which information, whilst being useful in following changes in phosphorus levels (e.g. during development of seeds, (Webster, 1928), gave no information about specific substances present.

A fractionation technique for demonstration of specific phosphorus-containing components of the acid soluble fraction of tissues was given by Le Page & Umbreit (1943) working with Thiobacillus thiooxidans. The procedure consisted of extraction with TCA and fractionation of the acid soluble components with barium at neutral pH, followed by subsequent analysis of phosphate esters in the extract. The technique, however, was not wholly successful when applied to plants. Albaum & Umbreit (1943) attempted the barium fractionation technique on developing oat seedlings and only a few compounds could be identified with certainty; inorganic orthophosphate, fructose-6-phosphate, hexose diphosphate, phytic acid and labile phosphorus which might be associated with ATP. Similar difficulties with the technique were found by Emerson, Stauffer & Umbreit (1944) working with Chlorella. These workers only identified inorganic phosphorus and hexose diphosphate with again evidence for the presence of ATP.

Further difficulties with the fractionation procedure were described for experiments on Euglena by Albaum et al. (1950). Labile phosphorus in tissue extracts was shown to be present in great excess of that known to be associated with the nucleotides. Thus if the lability of the phosphorus was relied on alone, Euglena would contain large amounts of an ATP-like substance. Further analysis showed that a portion of the highly labile phosphorus originated from inorganic pyrophosphate, and the remainder appeared to be inorganic metaphosphate. Using a modified technique, therefore, the workers showed the presence in Euglena of inorganic orthophosphate, metaphosphate and pyrophosphate, adenylic acid, ATP, ADP, DPN, glucose-1-phosphate, fructose-6-phosphate, hexosediphosphate, phosphoglyceric acid and riboflavin phosphate.

Albaum (1952) pointed out that precipitation techniques presume a knowledge of the constituents of the mixture before the results can be valid. From the point of view of methodology, a great advance was made by the combination of chromatography and autoradiography. These techniques did not require the large quantities of material previously necessary with precipitation techniques.

Knowledge of identity and interrelationships between phosphate esters has been expanded tremendously by the application of paper chromatography. The large number of phosphorylated compounds and the low concentrations of some of them require a method which is both sensitive and versatile. Paper chromatography meets these requirements. When dealing with paper chromatography of sugar phosphates special conditions are required for increasing the R_f values of these readily adsorbed compounds, which in the early chromatograms of Calvin & Benson (1949) showed almost no separation under conditions where the sugars,

hydroxyacids and amino acids were well separated.

As the importance of phosphate esters in plant metabolism became more obvious the chromatographic separations were improved. The greatest difficulties lay in the adsorption by impurities such as calcium, magnesium and iron which were present in the paper. The most pronounced demonstration of the adsorption effect was shown when attempts were made to chromatograph minute amounts of oxalic acid - C^{14}. This compound was progressively adsorbed until no more remained to move at its characteristic R_f. On washing the paper with oxalic acid, prior to chromatography, in order to elute or saturate immobile cations, oxalic acid and sugar phosphates chromatographed satisfactorily in phenol-water and butanol-acetic acid-water solvent systems. The acid wash also resulted in an acidic paper even after thorough rinsing with water and this gave higher R_f values for the acidic compounds.

Haynes and Isherwood (1949) used prewashes to avoid the effects caused by alkaline earth metals and by heavy metals. Alkaline earths were removed by pretreatment with hydrofluoric acid alone or by a mixture of hydrochloric and hydrofluoric acids. Heavy metal contamination was normally obviated by treatment with a solution of 8-hydroxyquinoline in aqueous alcohol followed by thorough washing with aqueous alcohol but, occasionally, when "oxine" treatment was not effective alone, saturation with hydrogen sulphide after oxine treatment was carried out.

The authors concluded that the most generally effective procedure seemed to be thorough washing with 2N acetic acid followed by washing with distilled water. Mortimer (1952) replaced the 8-hydroxyquinoline by 0.02% versene and claimed improved results.

A wide variety of solvent systems have been employed in the separation

of sugar phosphates. Haynes & Isherwood (1949) examined the properties of a number of solvents, the following being typical:

Acid, water immiscible solvent - 90 ml tertiary amyl alcohol/90 ml water/ 30 ml 90% formic acid

Acid, water miscible solvent - 80 ml tertiary butanol/20 ml water/4 g picric acid

Basic, water miscible solvent - 100 ml ethyl acetate/40 ml pyridine/100 ml H_2O

Benson et al. (1950) used phenol/water 72/28 w/w and butanol/proprionic acid/water 100/50/70 v/v/v for two-dimensional separations of plant extracts. Bandurski & Axelrod (1951) developed a two dimensional method for separation of phosphate esters in plant extracts. The solvent systems used were an acidic solvent composed of 80 ml methanol/15 ml 88% formic acid/5 ml H_2O and a basic solvent, 60 ml methanol/10 ml ammonium hydroxide (sp. gr. 0.9015)/30 ml H_2O. Chromatograms were developed at $2^{\circ}C$ with pre-equilibration of the paper in solvent vapour. Mortimer (1952) introduced a radical development in solvents when he replaced water with formamide.

The absolute R_f values of specific substances in the same solvent system can be affected by variations in temperature and solvent saturation of the atmosphere but only rarely do these variables affect relative R_f values. Mortimer (1952) used the concept of position constant, comparing the movement of a compound to that of orthophosphate.

The development of strong-base anion exchange resins produced another method for the separation of sugar phosphates. The great advantage of this method over paper chromatography is that much greater quantities of extracts can be separated easily. The capacity of the resin determines

the amount of material which can be separated but this is very high compared to that of cellulose adsorbants.

The principles of ion exchange chromatography are that a strong base anion resin in the chloride form acts as a stationary cation surrounded by a field of mobile chloride ions. The mobility of the chloride ions differs from that of ions such as glucose-6-phosphate and an equilibrium between the anions is established. Separation of polyacidic compounds, phosphoglyceric acid and hexosediphosphates, from the hexose monophosphates and orthophosphate was found to be relatively simple because of their great differences of adsorption on the resin. The two acid groups of PGA and the diphosphates decrease the ease with which the compound can be released from the resin. At low pH the carboxyl dissociation of PGA is supppressed and the elution properties are similar to those of a monophosphate.

Ion exchange separation of phosphorylated substances was developed by Cohn & Carter (1950) for nucleotides, Benson et al. (1950) for phosphoglyceric acid and hexose monophosphates and by Khym & Cohn (1953) who used the further modification of forming borate complexes of sugar monophosphates in dilute borate solutions.

With suitable modifications one might expect that any mixture of phosphates be separable.

Paper electrophoretic separation of sugar phosphates was reported by Schild & Buttenbruch (1953) but direct electrophoretic separation of plant phosphates has not been widely used since it does not yet offer any practical advantages over other methods.

Identification of Sugar Phosphates

The best possible evidence for the presence of sugar phosphates is chemical identification. Available methods for estimations of sugar phosphates require a prior knowledge of the components of the mixture and their possible reactions during the analysis. The problems of estimating particular esters in a plant extract are quite different from those met in determining the purity of a preparation or the components of an enzymatic reaction in which it is possible to predict what the products are. In this type of situation chromatographic separations give the selectivity needed to yield products pure enough for analysis by reasonably specific methods.

When dealing with paper chromatography absolute R_f values alone are hardly practical for identification of compounds in systems of many components because of variations in temperature and solvent saturation of atmosphere which can affect these values. However, measurement of relative R_f values and co-chromatography with pure standard substances are of much more value for identification purposes.

Physical and chemical properties of sugar phosphates which may be used for identification include salt solubilities, partition coefficients, optical activity, hydrolysis rates, acid strength, infra red spectra, colour reactions, enzymatic reactions and borate complexing characteristics.

The Influence of External Conditions on the Uptake of Phosphate

The uptake of phosphate from the surrounding medium is governed by many conditions, the physiological importance of which has not always been clearly established.

(a) Light

Since phosphorus plays an important role in photosynthesis, an influence of light on uptake of the element can be expected. Ketchum (1939a) found that the uptake of phosphate by P-deficient cells of "Nitzschia closterium" (= Phaedaetylum tricornutum) was always significantly greater in the light than in the dark. Phosphorus deficient cells of Chlorella pyrenoidosa likewise recovered more rapidly in the light than when kept in darkness (Ketchum, 1939b). However, with normal cells of C. pyrenoidosa, Emerson et al.(1944) could only demonstrate the promotion of phosphorus uptake by light when carbon dioxide was excluded, while Aranoff & Calvin (1948) failed to detect any influence of light on the rate of radiophosphorus uptake. Goodman et al. (1952) studied the incorporation of P^{32} in light and dark into phosphorus containing compounds in Scenedesmus obliquus. These workers observed rapid incorporation of P^{32} labelled phosphate into ATP in the dark and rapid incorporation of P^{32} into PGA in the light. From the rate of incorporation of P^{32} into ATP they concluded that under their conditions ATP was the first detectable product formed from inorganic phosphate. Kandler (1950) studied the transformation of orthophosphate during photosynthesis in Chlorella pyrenoidosa suspended in phosphate free medium. Kandler observed definite changes in cellular orthophosphate levels on illumination and when the cells were returned to darkness. Striking changes occurred in the first minute of illumination, the level of orthophosphate dropped sharply and rapidly recovered to a new steady-state level, the value of which was never higher than the steady-state level in darkness. When illumination was stopped, phosphate level rose to a maximum after a few minutes and then rapidly fell again to a new steady-state level.

Kamen & Spiegelman (1948) and Gest & Kamen (1948) using labelled phosphate showed in Chlorella and Rhodospirillum rubrum that the phosphate turnover in the TCA-insoluble fraction is greater in light than in dark and also that light stimulated the flow of low specific activity phosphate from acid insoluble phosphate into acid soluble phosphate with a simultaneous flow in the opposite direction. Kamen & Spiegelman considered their evidence as showing that "phosphorylation is mediated by light directly or indirectly in photosynthetic organisms". Simonis & Grube (1952) could not confirm the increase in P^{32} uptake into the TCA-insoluble fraction by leaves of Helodea densa. These workers observed an increase in P^{32} uptake into the TCA-soluble fraction in the light which was greater in the presence of CO_2 than in its absence. A further observation made by Simonis & Grube was that the inorganic phosphate level drops in the light. Wassink et al. (1951a) obtained similar results to those of Kamen & Spiegelman. In Chlorella vulgaris they found an increased conversion of TCA-soluble phosphate into TCA-insoluble phosphate in the light, particularly in the absence of CO_2. This conversion amounted to 30% of the phosphate content of the cells. Wassink et al. (1951b) noticed an increased uptake of orthophosphate by cells in the light in CO_2 absence. They fractionated (5 min. hydrolysis with NHCl at 100°C) the TCA-soluble phosphate into labile and stable forms and found the conversion of the labile fraction into the stable form reduced in the presence of CO_2. Addition of glucose had quantitatively the same effect as CO_2 in diminishing the conversion of labile into stable phosphate. Similar observations were made by Wintermans & Tjia (1952) who concluded that the extra phosphate which was taken up in CO_2 absence was largely stored in the labile phosphate compounds. Holzer (1951) found that a great

part of the TCA-soluble, 7 min. hydrolisable, phosphate in Chlorella is metaphosphate and has suggested that the ester phosphate fraction may be small. Holzer observed differences in the amount of metaphosphate in light and darkness, but it is not apparent from his data if the differences were significant.

Phosphorylation in cell free systems in light was investigated by Vishniac & Ochoa (1952) who incubated spinach chloroplast preparations with DPN, ATP, labelled phosphate and mitochondria. Phosphorus^{32} was incorporated into ATP, the incorporation depending on the complete system and light. The system was active with plant (mung bean) or animal (rat) mitochondria.

Kuhl (1957), Pirson & Kuhl (1958) found that in Hydrodactyon reticulatum cultured in intermittent light (12 hours light: 12 hours darkness) the total P content increased markedly during the light periods. This was mostly due to a reversible accumulation of inorganic phosphate, which in the succeeding dark periods was released from the plant.

Goodman et al. (1953) studied the distribution of radioactivity in Scenedesmus after incubation of the cells with P^{32} for 37 sec. in dark and 29 sec. in light. In darkness the ATP fraction contained 72% of the total activity and in light only 9.8%. These workers interpreted the results as indicating a faster phosphate turnover in light leading to a faster distribution into compounds other than ATP. Grube (1953), working with Elodea, demonstrated a much higher total uptake of P^{32} in light than in darkness but this was lower by only 30% when CO_2 was withdrawn, indicating that phosphate uptake depends only slightly on CO_2 assimilation. Simonis & Kating (1956) investigated the effect of light and glucose feeding on the uptake of P^{32} by Ankistrodesmus and found that

both treatments resulted in a higher P^{32} uptake and that light had an effect no greater than glucose feeding.

Although there are a number of experimental indications that light influences phosphorus uptake we are still unable to explain all the observed effects. Since active ion uptake is an energy requiring process, it seems reasonable to assume that light acts as an energy donor. However, there is no direct experimental evidence that the photochemical reactions of photosynthesis *per se* are involved in phosphate uptake.

(b) Phosphate concentration

Since, in nutrient solutions and usually in nature, phosphorus is exclusively present as phosphate, it would seem natural that the concentration of this compound in the medium would influence its rate of uptake by plants. Ketchum (1939a) showed that in "*Nitzschia closterium*" (= *Phaeodactylum tricornutum*) the absorption of phosphate per cell in the light depended on the phosphate concentration in the medium, whereas the phosphate uptake by cells deficient in phosphate was independent of phosphate concentration (Ketchum, 1939b). The phosphorus content of *Chlorella pyrenoidosa* and *Scenedesmus* (Gaffron's strain D_3) cells was found to depend strictly on the concentration of phosphate in the medium by Gest & Kamen (1948). Knauss & Porter (1954) showed that even if phosphate is supplied in excess, the phosphorus content per cell remains constant.

(c) Hydrogen ion concentration

The pH of the medium may affect the rate of phosphate uptake either by changing the principle ionic form of the phosphate or by directly altering the permeability of the cell membrane (Epstein, 1956). The first effect could be explained as a selective absorption of one of the three ionic forms

of phosphate; the latter effect could be associated with changes in the activity of enzymes assumed to be situated at the surface layer of the protoplasm and believed to be engaged in the absorption process. The selective absorption effect is supposed to be considerable, but the following data on the influence of external pH on phosphate absorption by algae are not conclusive. Scott (1945a) suspended C. pyrenoidosa cells for 1 hour in sodium phosphate solutions of various pH values and found that the intracellular phosphorus increased significantly more in alkaline solutions. Wintermans (1955), however, showed that in CO_2 absence, illuminated cells of C. vulgaris fixed phosphorus only in acid media (approx. pH 4). Badour (1959) in further studies with C. vulgaris found the influence of external pH on the uptake of phosphate, and subsequent distribution into several fractions, to be different in light and dark. MacKereth (1953) found the greatest uptake of phosphate by Asterionella formosa took place at values between pH 6 and pH 7.

(d) Other factors

A carbohydrate reservoir may be of importance in phosphorus uptake because the degradation of carbohydrate by oxidative phosphorylation can provide energy for the uptake process. Scott (1945b) noted that in Chlorella pyrenoidosa, potassium and phosphate were taken up in ratio of 1 atom K : 1 atom P; suggesting a chemical combination with some cell constituent. Badour (1959) showed that in C. vulgaris, both in light and darkness, absorption of phosphate was promoted by potassium. Krauss & Thomas (1954), working with Scenedesmus obliquus, observed that in early stages of growth in media deficient of micronutrients the P-content of the cells was appreciably higher than in normal cells. However, no determination

of the micronutrient responsible was made. For Nitzschia closterium (= Phaeodactylum tricornutum) nitrate concentration influences the rate of uptake of phosphate (Ketchum, 1939a).

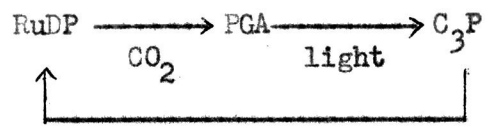
The presence of known organic compounds in the medium may also influence phosphate uptake. Wassink et al. (1951b) and Wintermans (1955) noted a suppression of phosphorus uptake by C. vulgaris in the light in the presence of 0.2% glucose. This suppression was explained by postulating that the assimilation of glucose and the incorporation of phosphate compete for energy generated by the photochemical reactions of photosynthesis. Simonis & Kating (1956) found a great difference between the rate of uptake of phosphorus in Ankistrodesmus braunii supplied with glucose (0.15%) before suspension in a phosphate containing medium and cells incubated with both glucose and phosphate simultaneously. In the first case, the rate of P-uptake was increased by 20%, in the second case, only a slight stimulation was observed. Jacobi (1950) observed an accelerated uptake of phosphate by A. braunii in the presence of glycolic acid (possibly one of the first products of photosynthesis), which suggests that glycolic acid or one of its derivatives, might act as a phosphate acceptor.

Precursor-Product Relationship

The criteria for the establishment of a precursor-product relationship have been given by Zilversmit et al. (1943) in the case where a steady-state conditions exists; i.e. the amount of substance (product) is constant with time, the rate of appearance (synthesis from precursor) equalling the rate of disappearance of product.

Excellent work into precursor-product relationships was carried out by

the Californian group of Bassham et al. (1954) investigating the labelling of various compounds with C^{14} in the pentose phosphate cycle in Scenedesmus and in soybean leaves. The plants were in steady-state photosynthesis. When they studied only ribulose diphosphate (RuDP), phosphoglycerate (PGA) and triose phosphate (C_3P), the CO_2 cycle could be represented as:



According to this scheme, RuDP would be the immediate precursor of PGA by way of a carboxylation reaction followed by a split to two PGA molecules. PGA in turn would be the source of triose phosphate for synthesis of cell material via reductive processes with light energy. It would be expected that in steady-state photosynthesis with labelled CO_2 sudden reduction of the CO_2 pressure would favour a short term increase in the effective concentration of RuDP and a corresponding fall in the concentration of PGA. Conversely, at constant CO_2 pressure, a decrease in light intensity would be expected to decrease the RuDP pool and increase the concentration of PGA. These expectations were borne out by the work of Wilson & Calvin (1955). The significance of these observations on the precursor-product relation of RuDP and PGA in the living cell lay in the inference that activation by light was not necessary for the carboxylation reaction. Quale et al. (1954) presented evidence to show that enzymatic carboxylation of RuDP could be carried out by cell free suspensions from Chlorella; as expected the reaction was not light sensitive. Thus, by indicating the kind of reactants to be looked for, the studies of the Californian group made possible the isolation of the

enzyme which is apparently responsible for the first step in CO_2 fixation in photosynthesis.

The tracer studies on CO_2 fixation in photosynthesis illustrate how precursor-product problems can be approached and how data from kinetic studies with labelled material can be used to infer a reaction sequence in intermediary metabolism.

The Significance of Tracer Methods for Biology

The central feature in tracer methodology is the preparation of labelled samples of substances involved in biological processes. It is obvious that the method makes contact with biology mainly at the biochemical and physiological level.

It should be noted that in growing organisms it is possible to study relationships between metabolites by non-tracer feeding experiments. In the steady state when growth has ceased, the only method available is a tracer method.

Before results from tracer-feeding experiments can be interpreted correctly, it is necessary to ensure that the label is adequate with regard to the following criteria (Kamen, 1957):

(i) the initial concentration of the tracer must be sufficient to withstand dilution during metabolism,

(ii) throughout metabolism the label must adhere to the particular molecule or portion of molecule with which it is originally incorporated,

(iii) abnormalities in metabolism must not be brought about through the action of the isotopic sample on the organism,

(iv) the half life of the isotope must be sufficiently long so that decay does not remove tracer faster than it can be extracted, characterised and assayed.

The Use of Radiophosphorus as a Tracer

The metabolic significance of phosphorus derives from its participation in esterification reactions. These reactions may be involved in both energy storage and for defining synthetic pathways. When using P^{32} as a tracer, interpretation of the results depends on the assumption that no exchange, other than that caused metabolically, occurs between phosphate esters and inorganic phosphate. The validity of this assumption has been demonstrated by many workers, among them Gourley (1952). Gourley mixed P^{32}-labelled inorganic phosphate with such esters as glucose-1-phosphate, adenylic acid, 2,3-diphosphoglyceric acid and ATP, under similar conditions to those in blood plasma. No exchange was observed. These researches, together with those of Hull (1941), who demonstrated with the use of labelled phosphates, that no exchange occurred between ortho-, meta- and pyrophosphate imply that any P^{32}-labelled compound present in plant extracts must be formed metabolically. Despite the behaviour mentioned above, it should be noted that carrier-free radioactive phosphorus tends to adsorb on suitable surfaces particularly where phosphate is present.

MATERIALS AND METHODS

(a) Materials

Laminaria digitata was collected from North Berwich at low tide throughout the year and stored in the laboratory in filtered sea water maintained at 10°C by means of a refrigerated cabinet. Illumination was provided by an overhead bank of six fluorescent tubes of the "warm white" type for 12 hours per day.

For all experiments algae were used in the form of discs, 1 cm in diameter, taken from all regions of the frond. Radioactive phosphorus to be incorporated into the plant material was added as inorganic phosphate to the bathing seawater. The isotope, supplied by the Radiochemical Centre, Amersham, was in the form of carrier free orthophosphate in dilute HCl.

(b) Phosphorus^{32} Incorporation into *L. digitata*

Discs cut from *L. digitata* fronds were immersed in filtered sea water containing approximately 100 microcuries of isotope per ml of sea water. Labelling was carried out for periods of time varying from two minutes to twenty four hours, as determined by the experimental procedure, and under various conditions of light and aeration. After removal from the radioactive medium, all discs were thoroughly washed with inactive, filtered sea water to remove excess radioactivity adhering to the surface of the tissue.

(c) Methods of Imposing Particular Experimental Conditions

The standard conditions adopted in the present work were to allow uptake of P^{32} to proceed for 12 hours in the light and 12 hours in darkness at 10°C with normal aeration. At the end of the experimental period the tissue was washed with filtered, inactive sea water and killed.

For experiments to show the effect of darkness on P^{32} uptake the tissue was kept in darkness for 12 hours prior to experimental procedure (dark adapted tissue), labelled in darkness for the required period, and later killed in a dark room, intermittent illumination being provided, when required for manipulative purposes, by an Ilford X darkroom safe light.

Similar dark adapted tissue was used in experiments to test the effect of light on phosphate uptake by L. digitata. In these experiments, P^{32} and illumination were given at the same instant. Illumination was provided by 3 fluorescent tubes of the Phillips TL 40 w. type, giving 350 ft. candles light intensity at a distance of 15 inches below an aluminium reflecting housing. In certain experiments the effect of a nitrogen atmosphere on P^{32} uptake was investigated. In this type of experiment, L. digitata discs were immersed in boiled, filtered sea water in a gas washing bottle (Dreschel bottle). During the whole of the experiment, including the dark adaptation period, nitrogen was bubbled through the sea water.

(d) Extraction of Phosphorus^{32} Labelled Compounds from L. digitata

Two methods of extracting P^{32} labelled substances from L. digitata were employed.

In the first of these methods the plant tissue, after washing with inactive sea water, was frozen with liquid nitrogen and ground to a fine powder with a mortar and pestle. The powder was then extracted by shaking with two 50 ml. volumes of 60% ethanol for a total of 120 minutes at room temperature.

In the second method the washed algal discs were plunged into boiling

60% ethanol and boiled for one minute. Extraction was contrived by shaking in the aqueous alcohol for 120 minutes.

After extraction by either of the above methods the alcoholic extract was centrifuged and the supernatant liquid evaporated to dryness under reduced pressure. The residue was then dissolved in a few drops of distilled water, the amount used depending on the procedure to be undertaken.

(e) Separation of Labelled Substances Present in an Alcoholic Extract of L. digitata

Various techniques were used for the separation and analysis of the P^{32} labelled compounds present in an alcoholic extract of Laminaria digitata.

(i) Paper Chromatography

The most extensively used technique was paper chromatography. Chromatograms were developed on various grades of paper and in a number of solvent systems.

Solvent systems used were as follows:

Bandurski and Axelrod Acidic and Basic Solvent Systems

The two dimensional solvent system of Bandurski & Axelrod (1951) was developed for the separation of phosphate esters in plant extracts. The compositions of the solvents are as follows:

Acidic Solvent 80 ml methanol/15 ml 88% formic acid/5 ml water

Basic Solvent 60 ml methanol/10 ml ammonium hydroxide (sp.gr. 0.9015)/30 ml water

The authors quote a temperature of 2°C for development of chromatograms. However, the use of the position constant concept (Mortimer, 1952), where instead of absolute R_f values being measured the relative distances moved by sample spots and some chosen reference compounds are compared, circumvents the need to develop at 2°C and also avoids errors due to temperature fluctuations and variations in solvent saturation of the

atmosphere.

In the present work, chromatograms were developed at room temperature on Whatman No. 1 paper in the ascending direction. The paper sheets (30 cm. x 30 cm.) were formed into cylinders and fastened with staples taking care that adjacent edges did not come into contact. The origin was situated at 2 cm. from the bottom edge of the paper.

Two dimensional separations were carried out developing first in the acidic solvent for 6 hrs. and secondly at right angles to the first run in the basic solvent (12 hrs.). The chromatograms were dried between the two runs.

The acidic solvent system was found to give a good separation of the P^{32} labelled substances in an alcoholic extract of L. digitata in 6 hrs. at room temperature and this solvent was extensively used.

Cowgill's Solvent

The solvent consists of 70 ml 95% ethanol/1 ml formic acid/29 ml water. Ascending and descending chromatography was carried out in this solvent. Good separations of the radioactive substances present in alcoholic extracts were obtained on Whatman No. 1 paper developed for 16 hrs. in the descending direction at room temperature.

Ebel's Acidic Solvent System

General methods for differential analysis of mixtures of condensed phosphates have been developed on the basis of Ebel's ascending paper chromatographic techniques. In the present work, use is made of Ebel's acidic solvent system and the fact that the plot of chain length of condensed polyphosphates against the R_f value in the acidic solvent results in a straight line graph (Grunze & Thilo, 1953).

Ebel's acidic solvent consisted of 750 ml isopropyl alcohol, a solution

of 50 gm TCA in 250 ml water and 2.5 ml concentrated ammonia.

Chromatograms were developed on 25 cm x 25 cm Whatman No. 1 chromatography paper formed into cylinders and run for 6 hours. Pre-treatment of the chromatograms consisted of allowing them to be in contact with the solvent vapour for 45 mins.

Standard phosphates were run in all solvents for comparison with the chromatographic properties of all the radioactive substances present in the untreated alcoholic extract of L. digitata.

For collection of samples of the separated phosphorus^{32} containing substances in the L. digitata extract, Whatman 3 mm chromatography paper was used since this has a higher loading capacity than Whatman No. 1 paper.

(ii) Ion Exchange Chromatography

Separation of untreated alcoholic extract from P^{32} labelled L. digitata was accomplished on Amberlite anion exchange resin. The strong base anion resin IRA 400 was employed. Formate, chloride and hydroxide forms of the resin were used.

Ion exchange resin was kept in N.HCOOH, N.HCl, or N.NaOH overnight to obtain the form required. Columns (10 cm x 1 cm) were prepared and a sample of extract added to the top. Solvents including water, NaCl, NH_4OH, NH_4Cl, HCl and formic acid, were used to develop the column, liquid fractions from which were monitored with a F10 flow counter, ratemeter and Honeywell-Brown recorder. Flow rates of approximately 1 ml per minute were maintained and 5 ml samples obtained by means of an automatic sample collector. Samples containing peak activities were concentrated by evaporation under reduced pressure and subjected to further analysis.

(iii) Paper Electrophoresis

The usually adopted procedure was to subject to electrophoresis crude

alcoholic extract of P^{32} labelled L. digitata on 21 cm x 4 cm strips of Whatman 3 mm paper. Electrophoresis was carried out in a pH 4 buffer (M/20 potassium hydrogen phthalate) at 300v, 10-15 mA, for 90 minutes.

(f) Location of Phosphorylated Compounds on Paper

Phosphorus containing substances, separated by paper chromatography or paper electrophoresis, were located either by the reaction of phosphate groups with acid ammonium molybdate or by detection of radioactivity.

The chemical detection of phosphate containing regions on paper was achieved by dipping the paper in an acid ammonium molybdate reagent (Burrows, 1952) followed by illumination with an ultraviolet light source. The ammonium molybdate reagent consists of 1 g of ammonium molybdate in 8 ml water, diluted to 100 ml with acetone. The U.V. source used was a Phillips 300 w germicidal lamp.

Autoradiographs of chromatograms and electrophoretograms were prepared with Ilfex Industrial-G X-Ray Film for the detection of regions containing radioactive phosphorus.

A quick convenient method was evolved for location of P^{32} containing areas on paper strips. The paper strip was attached to the chart of a Honeywell-Brown recorder below the pen. As the chart ran, the strip was carried past a collimated EHM2 counter. The counter was connected to a probe unit and ratemeter, whose output actuated the pen. By careful marking of the positions of the pen and Geiger-Müller tube at the start and finish of the scan, the strip and record were aligned and radioactive regions accurately located. Good correlation of graph peaks, so obtained, with positions of radioactivity detected by autoradiography, was achieved.

(g) Estimation of Inorganic Phosphate in Laminaria digitata extracts

The inorganic phosphate content in plant extracts was estimated by a colorimetric method. The reagents used were:

Reducing reagent - 50 mg N-phenyl-p-phenylene diamine monohydrochloride in 100 ml 1% $NaHSO_3$

Ammonium molybdate - 5% w/v in 15% v/v H_2SO_4

Perchloric acid - 60% v/v in distilled water

The procedure was to add a known volume of extract to a 10 ml volumetric flask, followed by 0.5 ml perchloric acid, 0.4 ml ammonium molybdate and 4 ml reducing agent. The solution was made up to the mark with distilled water and allowed to stand at room temperature for 10 minutes, after which the colour was estimated in a EEL colorimeter provided with an Ilford 607 filter. A calibration curve using standard phosphate solutions was prepared (Fig. i).

(h) Estimation of Relative Labelling in Phosphorus^{32} Containing Components of Alcoholic Extract of Laminaria digitata

The radioactivity in P^{32} labelled compounds present in a 60% aqueous alcohol extract of plant tissue was estimated after chromatography and preparation of autoradiographs.

The P^{32} containing regions were located on the chromatogram, with the aid of the autoradiograph, cut out and digested in 5 ml of perchloric acid which was then made up to a 10 ml volume. The sample was then counted in a M6 liquid counter (20th Century Electronics). Corrections were made for background and lost counts to the counts per minute determined by standard scaling units (A.E.R.E. type 1009D).

/

Inorganic Phosphate Estimation

Calibration Curve

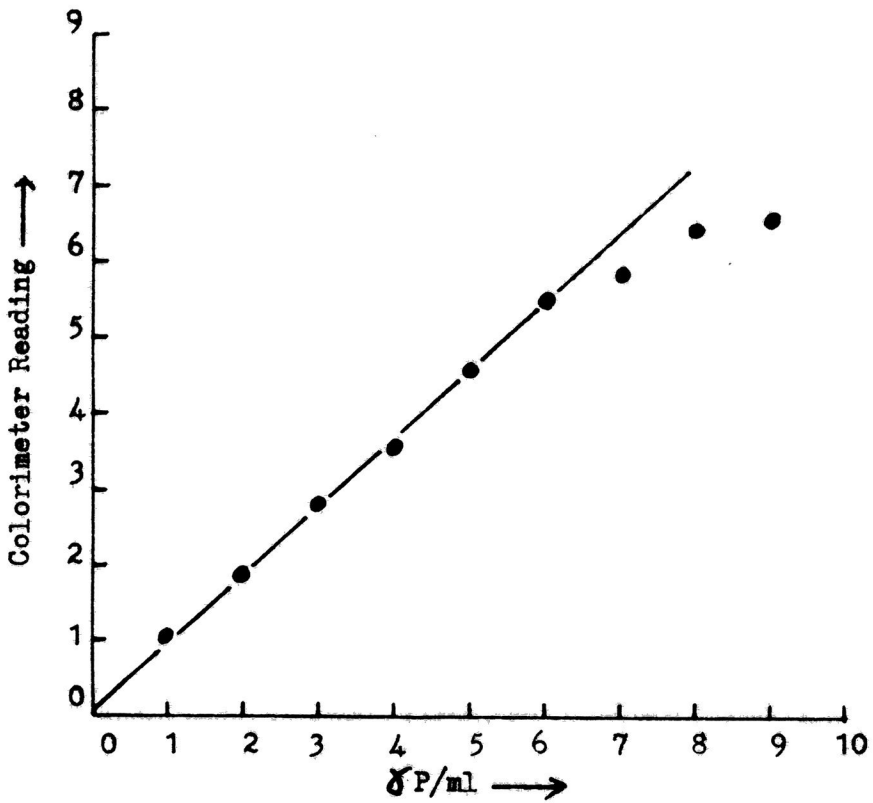


Fig. 1

(i) Estimation of 60% Aqueous Alcohol Extractable and Unextractable Inorganic Phosphate in Laminaria digitata

(i) Extractable Inorganic Phosphate

The orthophosphate level in the alcoholic extract of L. digitata can be estimated by the colorimetric method quoted above. Since a known weight of tissue was extracted with a known volume of solvent, the extractable orthophosphate in micrograms per gram fresh weight of sea weed can be calculated, i.e.

$$\frac{\text{micrograms of phosphate/ml extract x total volume solvent}}{\text{fresh weight in gms of tissue extracted}}$$

(ii) Total Extractable Phosphate

This is an estimation of the extractable orthophosphate plus other phosphorylated compounds, i.e. organic phosphate and any pyrophosphate or metaphosphate. The organic phosphates and meta-, or pyrophosphate are hydrolysed to produce orthophosphate under the wet ashing conditions employed.

A known volume of extract was evaporated to dryness and the residue wet ashed in a 50/50 (v/v) HNO_3/H_2SO_4 mixture on a hot plate. The sample was then neutralised with NaOH and made up to a known volume. Orthophosphate present was then estimated by the usual colorimetric method. This result was used for the calculation of the total extractable phosphate per gram fresh weight of tissue, i.e.

$$\frac{\text{micrograms of phosphate/ml neutralised sample} \times \frac{\text{total volume neutralised sample}}{\text{volume of extract wet ashed}} \times \text{total volume of solvent used for extraction}}{\text{fresh weight of tissue in gms}}$$

(iii) Total Unextractable Phosphate

A sample of previously extracted weed was taken and blotted dry. The weighed sample was then wet ashed as above, the residue neutralised and made up to a known volume. Orthophosphate in this sample was then estimated and the total unextractable phosphate per gram fresh weight of tissue calculated, i.e.

$$\frac{\text{micrograms of phosphate/ml neutralised sample} \times \text{total volume neutralised sample}}{\text{fresh weight in gms of tissue wet ashed}}$$

(j) 60% Aqueous Alcohol Fractionation of P^{32} Present in L. digitata

The radioactivity extracted by 60% aqueous alcohol and the unextractable P^{32} were calculated in the following way.

Extractable radioactivity. A known volume of extract was counted in a liquid counter and the counts per minute per gram fresh weight of tissue calculated, i.e.

$$\frac{\text{cpm/ml extract} \times \text{volume of extract}}{\text{fresh weight in grams of tissue extracted}}$$

Unextractable radioactivity. The unextractable radioactivity was estimated by wet ashing a previously extracted sample of tissue, making the residue up to a known volume and counting a sample of known volume in a liquid counter. From this figure the unextractable cpm/gm fresh weight can be calculated, i.e.

$$\frac{\text{cpm/ml wet ashed sample} \times \text{total volume wet ashed sample}}{\text{fresh weight in gms of tissue wet ashed}}$$

(k) Elution of Substances Separated by Paper Chromatography

In order to transfer substances separated by chromatography from the original paper to a fresh sample of chromatography paper the following technique was employed:

A rectangular strip, containing the substance under investigation, was cut from the original chromatogram and shaped to a point at one end. The paper strip was then clamped between two perspex blocks (which were just shorter than the paper strip) with the pointed tip projecting beyond the perspex. The perspex blocks, acting as a support for the paper strip, were placed in distilled water which just covered the lower edge of the paper strip. The pointed end of the sample strip was applied, from below, to the fresh sample of chromatography paper. Warm air from a hair drier was directed on to the upper surface of the chromatography paper. The sample was thus passed from the original chromatogram to fresh chromatography paper by capillary transfer and evaporation.

(l) Spectrophotometry and Ultraviolet Fluorescence

Spectrophotometry was carried out using a Perkin Elmer 137 U.V. spectrophotometer and examination for fluorescence was performed under a "Chromatolite" U.V. lamp with visible cut-off filter. Samples of extract and unstained chromatograms were examined in this way.

Print from Autoradiograph after Chromatography of

$KH_2PO_4 + P^{32}$ and $K_2HPO_4 + P^{32}$

(for explanation see text)

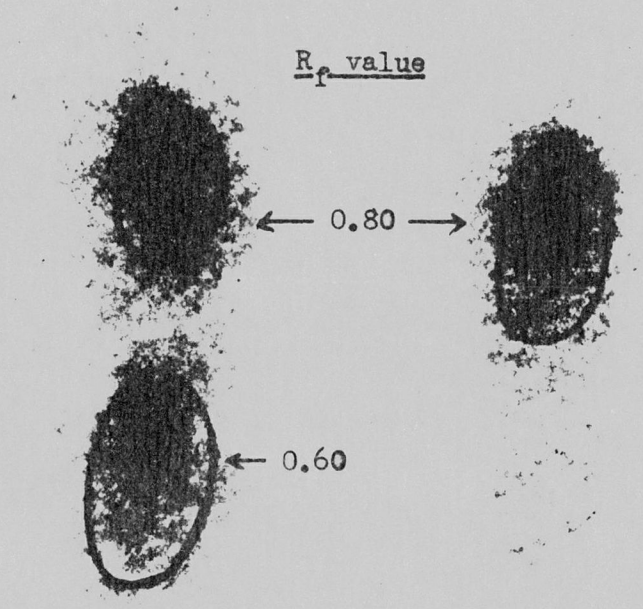


Fig. ii

RESULTS

(a) The Exchange of Carrier Free Radioactive Orthophosphate with Other Phosphorylated Substances

It was shown by the researches of Gourley (1952) and Hull (1941) that no exchange of radioactive label took place between labelled orthophosphate and phosphate esters or between meta-, pyro- and orthophosphate. However, in the present work, radioactive phosphate was administered in the carrier free form and in this state P^{32} will absorb on suitable surfaces, particularly where phosphate is present. That this is indeed the case was shown by chromatography of mixtures of carrier free P^{32} and other phosphates. Unlabelled phosphates were located with ammonium molybdate and radioactive regions detected by autoradiography. It was observed from these investigations that, in the regions showing a positive reaction with the stain, P^{32} was also present.

Fig. ii shows the results obtained by autoradiography when P^{32} was chromatographed as a mixture with potassium dihydrogen orthophosphate and dipotassium hydrogen orthophosphate in Bandurski and Axelrod's acidic solvent for six hours at room temperature. Under these conditions K_2HPO_4 shows a positive staining reaction at $R_f = 0.80$ and KH_2PO_4 shows positive staining at $R_f = 0.80$ and $R_f = 0.60$. These R_f values correspond with those of the radioactive areas. Under the same conditions carrier free orthophosphate alone has an $R_f = 0.80$.

Further evidence for the adsorption of P^{32} on to phosphorylated substances can be seen when P^{32} (carrier free) and crude extract are chromatographed as a mixture. There is an increase in the radioactivity as detected by autoradiography, not just in the orthophosphate region but in all regions having phosphate present.

These results should be borne in mind when interpreting the results of extraction of labelled phosphates from L. digitata, since it is conceivable that some of the P^{32} regions observed might not be formed by metabolism but by an exchange process.

That it is possible for at least two of the typical P^{32} containing regions, detected by chromatography and autoradiography of an untreated alcoholic extract of labelled L. digitata, to be formed by an exchange process rather than by metabolism is shown by the following work.

A sample of weed was frozen under liquid nitrogen and then allowed to thaw, after which it was labelled with carrier-free P^{32} under the usual conditions. After labelling the tissue was extracted with 60% aqueous ethanol in the manner described above.

A second sample of L. digitata was frozen with liquid nitrogen and an alcoholic extraction carried out. The extract was then incubated with P^{32} (carrier-free) for 24 hrs.

On chromatography and autoradiography of both of the above samples the radioactivity was seen to be located at regions thought to be orthophosphate and pyrophosphate. It should be noted that the freezing technique kills the weed if photosynthesis is taken as a criterion of life, since after freezing and thawing the tissue will no longer carry out the oxygen evolutionary stage of photosynthesis as shown manometrically.

(b) Comparison of Trichloracetic Acid and Aqueous Alcohol Extracts from Laminaria digitata

In order to compare the results of extraction of P^{32} containing substances by ethyl alcohol and trichloracetic acid from L. digitata which had been incorporating the isotope for 24 hours, under the standard conditions referred to above, the following experiment was performed. Extracts were

prepared with 60% aqueous alcohol and with 10% TCA. Both extracts were prepared in the same way by freezing a known amount of tissue and shaking the powder resulting from grinding in two volumes (50 ml each volume) of 10% TCA or 60% aqueous ethyl alcohol for a total of 120 minutes.

Comparisons were made of the R_f and Rp^{32} values of radioactive components (the Rp^{32} value being defined as the distance moved by a particular substance relative to the distance moved by P^{32} (carrier-free) in the same solvent and in the same time), proportion of total inorganic phosphorus extracted and proportion of total radioactivity extracted by each solvent.

Chromatography was carried out in the acidic solvent of Bandurski and Axelrod for 6 hours in the ascending direction and autoradiographs prepared from the chromatograms. Table II shows typical results for the R_f and Rp^{32} values of labelled substances found in the extracts.

Table II

R_f and Rp^{32} values of phosphorylated compounds present in alcoholic and TCA extracts of L. digitata chromatographed in Bandurski and Axelrod acidic solvent system at room temperature

60% aqueous alcohol extraction		TCA extraction	
R_f	Rp^{32}	R_f	Rp^{32}
0.52	0.65	0.52	0.65
0.63	0.79	0.61	0.76
0.68	0.87	0.69	0.86
0.74	0.93	0.73	0.91
0.81	1.0	0.81	1.01

Table III shows the phosphate levels in the extracts and the phosphate remaining in the tissue after extraction.

Table III

Comparison of extractable and unextractable phosphate levels in 10% TCA and aqueous alcoholic extracts of L. digitata

	Aqueous alcoholic extraction (micrograms phosphate/gm fresh weight of tissue)	10% TCA extraction (micrograms phosphate/gm fresh weight of tissue)
Extractable orthophosphate	17.99	13.32
Total extractable phosphate	93.3	112.68
Total unextractable phosphate	400.0	330.5

The radioactivity extracted by aqueous ethyl alcohol and by 10% aqueous TCA from P^{32}-labelled L. digitata is shown in Table IV.

Table IV

Comparison of extractable and unextractable phosphorus^{32} in aqueous alcoholic and 10% TCA extractions of L. digitata

	Aqueous alcoholic extraction (cpm/gm fresh weight)	10% TCA extraction (cpm/gm fresh weight)
Extractable P^{32}	120936	147686
Unextractable P^{32}	363333	233333

The relative labelling of the P^{32} labelled substances was estimated by cutting out radioactive areas, digesting the paper in perchloric acid and counting the samples in a liquid counter. The results are shown in Table V.

Print from Autoradiograph after Chromatography in Bandurski and Axelrod's Acidic Solvent of 10% Aqueous TCA and 60% Aqueous Ethyl Alcohol Extracts from L. digitata containing P^{32}

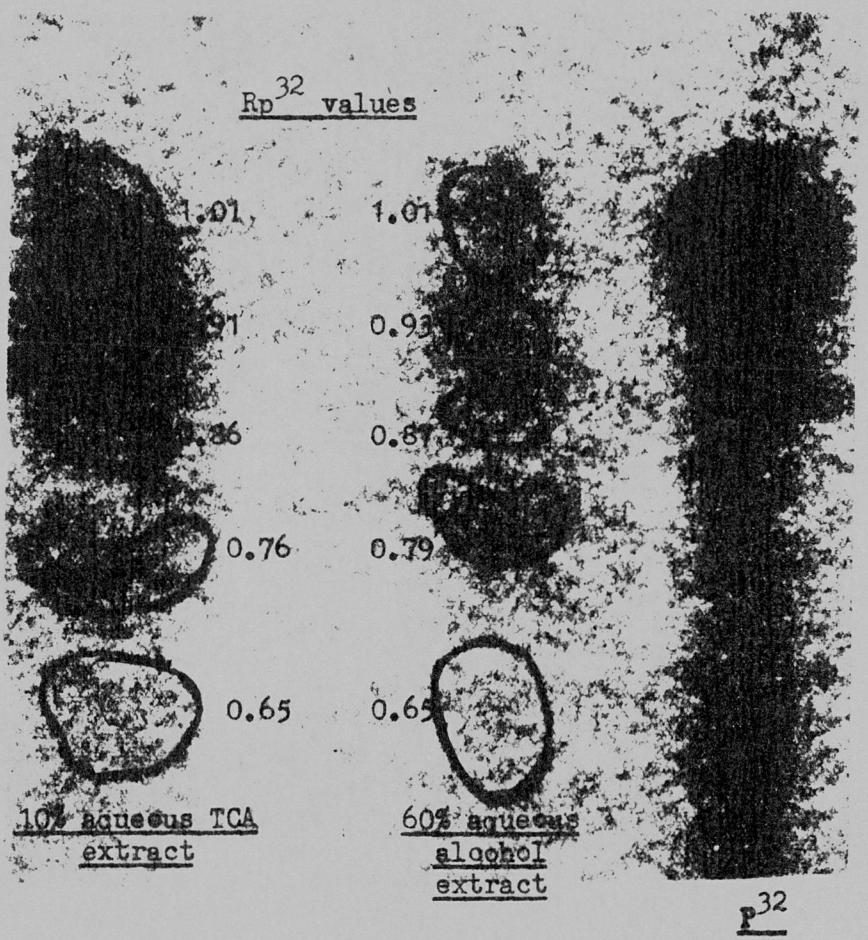


Fig. iii

Table V

Relative labelling of P^{32} containing regions after chromatography of alcoholic and acidic extracts of L. digitata in the Bandurski and Axelrod acidic solvent

Aqueous alcoholic extract		10% TCA extraction	
Rp^{32} value	% activity	Rp^{32} value	% activity
0.65	9.9	0.65	11.2
0.79	21.5	0.76	21.4
0.87	21.1	0.86	15.1
0.93	22.0	0.91	20.3
1.0	25.5	1.01	32.0

A print of an autoradiograph of the TCA and alcohol extracts after chromatography in the Bandurski and Axelrod acidic solvent is shown in Fig. iii. A photograph of a typical chromatogram showing regions with a positive reaction to ammonium molybdate reagent is illustrated in Fig. iv.

Discussion

By comparing the number and chromatographic properties of P^{32} containing substances extracted by 60% aqueous alcohol and 10% TCA, it can be seen that both solvents extract the same compounds. Since it has not yet been determined whether the orthophosphate present in a TCA extraction is all intracellular orthophosphate or whether some of this inorganic phosphate results from hydrolysis of organic forms during extraction, it would seem better, from the point of view of determining the distribution of P^{32} in compounds present in a plant extract, to avoid any errors arising from this source if at all possible. Also, from examination of Table V, it appears that increased orthophosphate is detected in plant material extracted with 10% aqueous TCA and it is possible that in this case organic phosphates have been broken down during extraction. Thus in the present work, 60% ethyl alcohol is preferred as the standard solvent. It has the additional advantage of

Regions Having Positive Reaction with Acid Ammonium Molybdate After Chromatography of 10% Aqueous TCA and 60% Aqueous Ethyl Alcohol Extracts of L. digitata in the Acidic Solvent of Bandurski and Axelrod

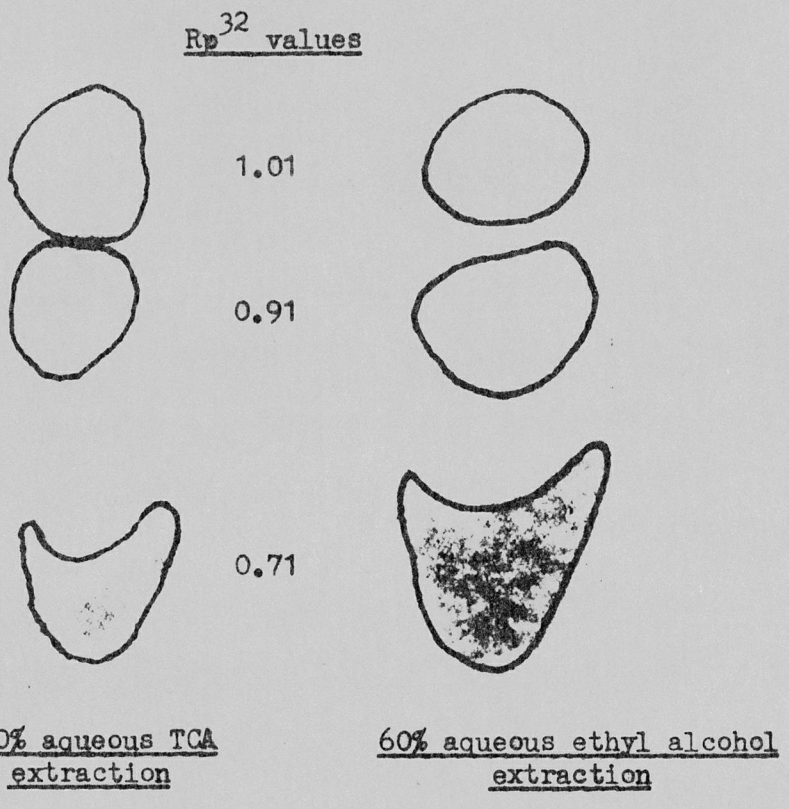


Fig. iv

minimising the extraction from the weed residue of alginic acid which gives a viscous solution difficult to concentrate by evaporation.

It should also be noted at this stage that the chromatographic and autoradiographic pictures did not differ according to the type of solvent used for extraction, nor according to the method of concentration of the extract. The same radioactive regions as detected above were shown to be present on extraction with any of the following solvents; 80% aqueous ethanol; 60% aqueous ethanol, boiling water or cold water. Alternative conditions of concentration such as freeze drying or concentration under reduced pressure with argon being drawn through the apparatus in place of air were also shown to be without influence on the chromatographic and autoradiographic pictures obtained, but generally the temperature of the extract during concentration was maintained below 25°C to minimise possible changes in composition of the extract during concentration.

(c) Number and Nature of Phosphorylated Compounds Present in a 60% Aqueous Alcohol Extract of L. digitata

(i) Examination by Paper Chromatography

60% aqueous alcohol extracts of Laminaria digitata which had incorporated radioactive phosphorus under the standard conditions quoted above were examined in a number of chromatographic solvents. Standard phosphates were chromatographed in the same solvents to obtain some indication of the possible nature of P^{32} containing substances extracted by aqueous alcohol. The results of this investigation will be given under the headings of the solvent systems employed.

Cowgill's Solvent Descending chromatography was carried out for 16 hours at room temperature on Whatman No. 1 chromatography paper. Table VI shows the R_f values and Rp^{32} values of substances present in the alcoholic extract when chromatographed under the stated conditions.

The Results of Chromatography of Crude 60% Aqueous Ethyl Alcohol Extract from L. digitata in Cowgill's Solvent

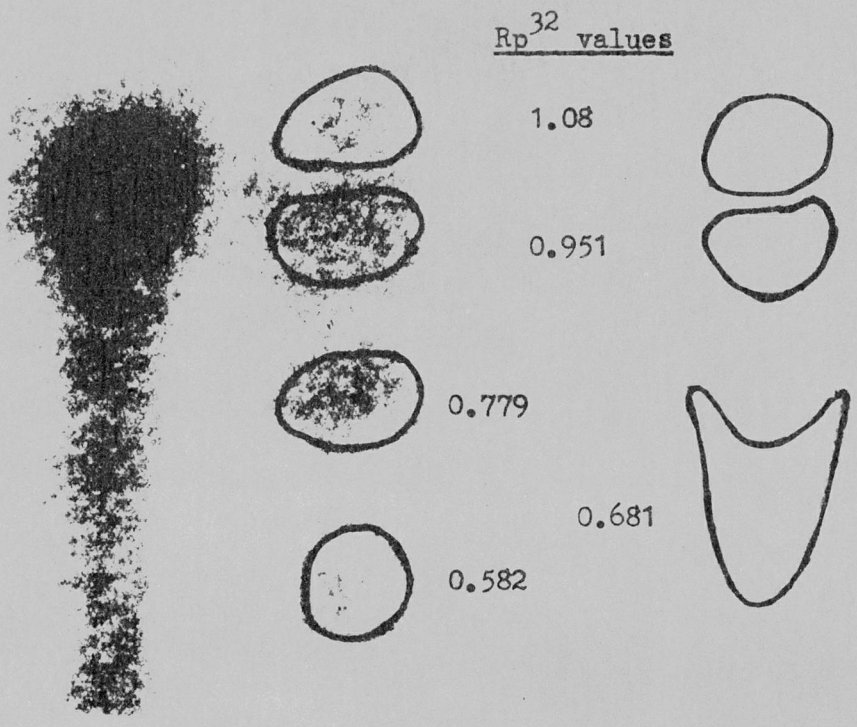


Fig. v

Table VI

R_f and Rp^{32} values of standard phosphates and P^{32} labelled substances present in an alcoholic extract of L. digitata chromatographed in Cowgill's solvent

Substances	R_f	Rp^{32}
NAD $NADH_2$	0.184	0.298
NADP	0.168	0.28
ATP	0.27 0.541 0.598	0.452 0.904 1.013
ADP	0.311 0.541 0.598	0.452 0.904 1.013
F-6-P	0.533	0.890
G-1-P G-6-P	0.504	0.84
FDP	0.544	0.906
$P^{32}0$	0.60	1.0
Creatine phosphate	0.576	
UDP	0.288	
UTP	0.201	
M-1-P	0.636	1.06
Extract	0.425 0.569 0.695 0.79	0.582 0.779 0.951 1.08

A photograph of a typical ammonium molybdate stained chromatogram of the crude extract and an autoradiograph from such a chromatogram is presented in Fig. v.

Bandurski and Axelrod Acidic and Basic Solvent Systems

The acidic and basic solvents used as a two dimensional system are reported as having been useful in the separation of phosphate esters in plant extracts.

Two Dimensional Separation of Alcoholic Extract from L. digitata Using the Bandurski and Axelrod Acidic and Basic Solvent Systems

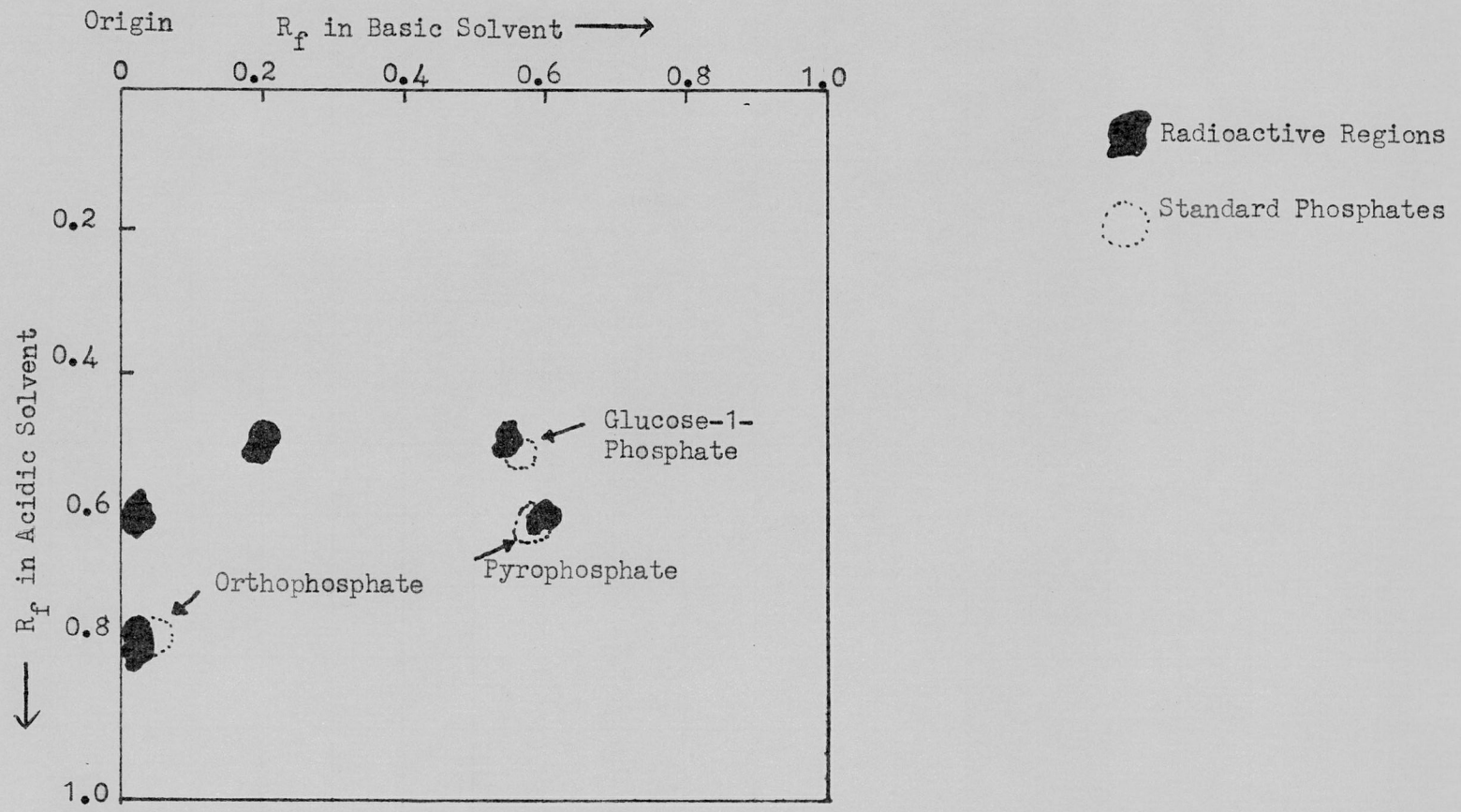


Fig. vi

In the present work chromatography was carried out at room temperature for 6 hours in each solvent. Chromatograms were developed in the ascending direction on Whatman No. 1 paper.

A diagram of a two dimensional separation of L. digitata extract using the above solvents is shown in Fig. vi. Discrete radioactive areas after chromatography of the crude extract were detected by autoradiography. Phosphate standards were detected by dipping in the ammonium molybdate reagent.

Table VII shows the R_f values of some standard phosphates together with the values for P^{32} labelled substances present in an alcoholic extract from L. digitata in the acidic solvent and in the basic solvent.

Table VII

R_f values in the acidic and alkaline solvents of Bandurski and Axelrod of standard phosphates and the P^{32} containing substances present in crude alcoholic extract of L. digitata

	R_f value	
Substance	Acidic solvent	Basic solvent
3PGA	0.23	no movement
ATP	0.23	0.48
ADP	0.35	0.46
PM	0.60	no movement
G-1-P	0.52	0.57
G-6-P	0.54	0.70
FDP	0.55	0.66
PP	0.60	0.58
R-5-P	0.63	0.63
M-6-P	0.65	0.71
$P^{32}O$	0.80	no movement
Extract	0.24	(no movement
	0.34	(
	0.45	(0.2
	0.55	(
	0.61	(
	0.82	(0.61

Autoradiograph of Crude Alcoholic Extract from L. digitata Showing P^{32} Containing Regions Resulting After Chromatography in Ebel's Solvent

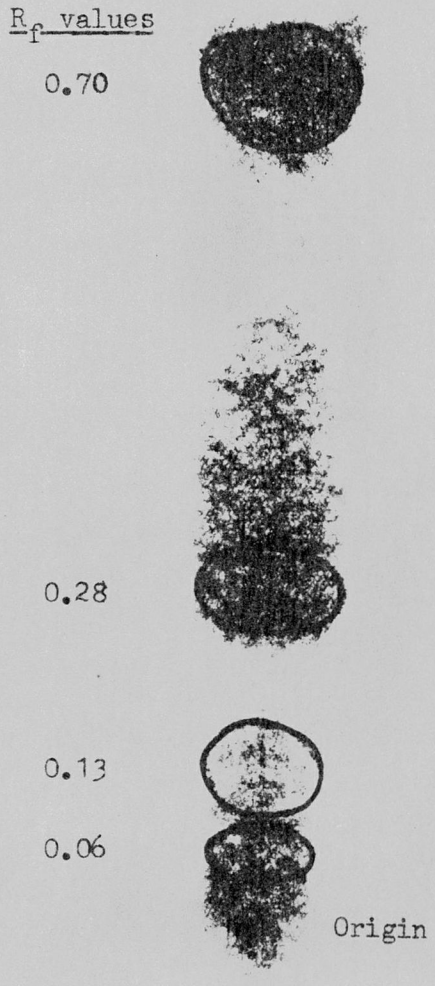


Fig. vii

Chromatography in Ebel's Acidic Solvent

Alcoholic extract from L. digitata which had incorporated P^{32} for 24 hours under standard conditions was chromatographed in Ebel's acidic solvent. The conditions under which chromatograms were developed are described above.

Chromatography followed by autoradiography showed three main regions containing P^{32} (Fig. vii).

The logarithms of the R_f's of the main regions of radioactivity are shown plotted on the graph of log R_f against chain length of polyphosphate (Fig. viii). The R_f values (quoted below, Table VIII) of the polyphosphates were taken from Karl-Kroupa (1956).

Table VIII

Phosphate	Chain Length	Acidic R_f	$\log_{10} R_f$(x100)	Mean log R_f(x100)
ortho	1	0.68 - 0.73	1.8325 - 1.8633	1.8479
pyro	2	0.42 - 0.48	1.6232 - 1.6812	1.6522
Tri	3	0.21 - 0.33	1.3222 - 1.5185	1.4204
Tetra	4	0.11 - 0.22	1.0414 - 1.3424	1.1919
Penta	5	0.06 - 0.14	0.7782 - 1.1461	0.9622
Hexa	6	0.09	0.9542	0.9542
Hepta	7	0.02 - 0.06	0.3010 - 0.7782	0.5396
Trimeta		0.14 - 0.21	1.1461 - 1.3222	1.2342
Tetrameta		0.08 - 0.11	0.9031 - 1.0414	0.9723

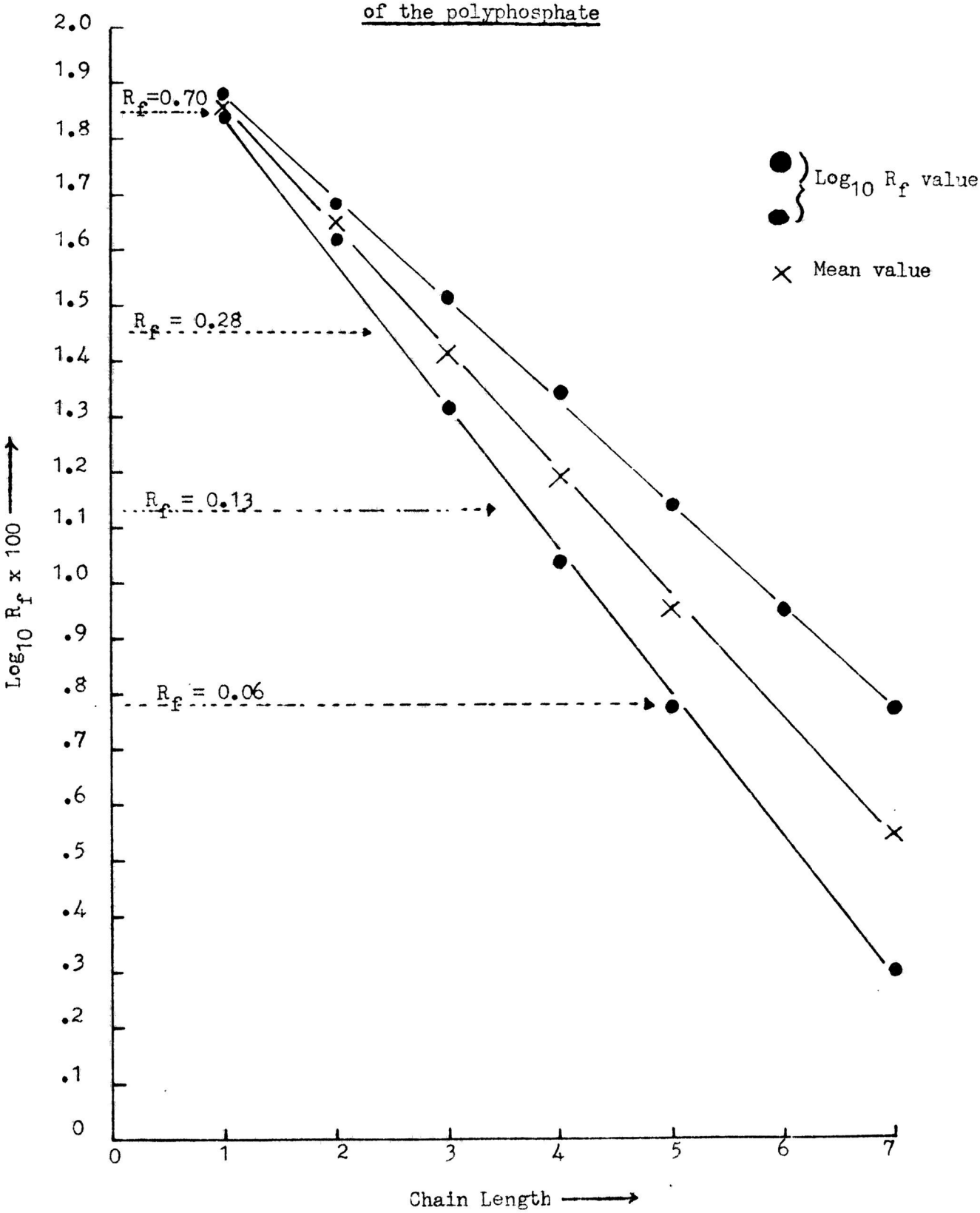
Crude extract showed regions of activity at $R_f = 0.70$; $R_f = 0.28$; $R_f = 0.13$ and $R_f = 0.06$.

On staining with acid ammonium molybdate the only regions giving the positive reaction for phosphates were at $R_f = 0.70$ and $R_f = 0.22$.

Pyrophosphate, when used as a standard in the present work, had an $R_f = 0.33$. Metaphosphate showed three positive regions when stained with ammonium molybdate, $R_f = 0.69$; $R_f = 0.32$ and $R_f = 0.21$.

Fig. viii

Graph of $\log_{10} R_f$ polyphosphate v chain length of the polyphosphate



P^{32}, as supplied by the Radiochemical Centre, Amersham when used as a standard in Ebel's acidic solvent has $R_f = 0.70$.

Consideration of these results would indicate that the radioactive region with $R_f = 0.70$ is orthophosphate. The P^{32} containing region with $R_f = 0.28$ could be pyrophosphate. On the evidence from this investigation alone, no useful deductions can be made as to the nature of the radioactive peaks at $R_f = 0.13$ and $R_f = 0.06$.

The chromatographic pattern obtained from the crude extract stained with ammonium molybdate is different from the pattern of radioactivity as shown by autoradiography. The region $R_f = 0.70$ shows the presence of P^{32} and gives a positive reaction with the stain. No other radioactive region shows the positive staining reaction. There is a strong 'molybdenum blue' region at $R_f = 0.22$. From the R_f values of the standard metaphosphate it can be seen that this $R_f = 0.22$ region corresponds to one of the three regions giving positive staining reaction on chromatography of metaphosphate. Since the remaining two stained regions of the metaphosphate have R_f values of 0.69 and 0.32, these regions most probably correspond to orthophosphate and pyrophosphate respectively. From the results in Table VIII it may be that the $R_f = 0.22$ region is trimetaphosphate. If this is so the trimetaphosphate in the plant is not radioactive under the conditions of labelling in this work.

Correlation of Chromatography in Ebel's Acidic Solvent and Bandurski and Axelrod's Acidic Solvent System

Untreated 60% alcohol extract of L. digitata was chromatographed in Ebel's acidic solvent and the main radioactive regions eluted with water for

rechromatography in the acidic solvent of Bandurski and Axelrod. The detection of phosphorylated compounds was by autoradiography.

The region R_f = 0.70 in Ebel's solvent was eluted and rechromatographed in the Bandurski and Axelrod solvent in the ascending direction at room temperature for six hours along with a sample of crude extract. The R_f values of radioactive regions observed are shown in Table IX.

Table IX

R_f values in Bandurski and Axelrod acidic solvent of P^{32} labelled substances present in alcoholic extract from L. digitata and the compound R_f = 0.70 eluted after chromatography in Ebel's acidic solvent

R_f values	
Untreated Extract	Eluted Substance
0.23	-
0.34	-
0.45	-
0.58	-
0.64	-
0.80	0.80

The radioactive region R_f = 0.28 resulting from chromatography of the crude extract in Ebel's solvent was treated in the same manner as the region R_f = 0.70 and the results are presented in Table X.

Table X

R_f values in Bandurski and Axelrod acidic solvent of P^{32} containing substances present in untreated 60% alcoholic extract of L. digitata and substance R_f = 0.28 eluted from chromatography of the extract in Ebel's acidic solvent

R_f values	
Untreated Extract	Eluted Substance
25	-
36	-
45	-
55	55
64	64
82	82

The remaining radioactive region, R_f values in Ebel's acidic solvent = 0.6, 0.13, was rechromatographed in the same manner as the other phosphorylated regions and the results are shown in Table XI.

Table XI

R_f values in Bandurski and Axelrod acid solvent of P^{32} containing substances present in 60% alcoholic extract of L. digitata and substances R_f = 0.13, R_f = 0.06 eluted after chromatography of the extract in Ebel's acidic solvent

R_f values	
Untreated Extract	Eluted Substance
25	-
36	-
45	45
55	56
64	63
82	81

Hydrolysis of untreated 60% aqueous ethanol extract of Laminaria digitata

Hydrolysis of a 60% aqueous ethanol extract of L. digitata, which had incorporated radioactive phosphorus under standard conditions, was undertaken with the object of providing possible evidence for the identification of P^{32} containing substances present in the extract.

Overnight hydrolysis of the extract was carried out at 100°C with N.HCl and with N.NaOH. The acid and alkaline hydrolysates were then subjected to chromatography in a solvent consisting of butanol/acetic acid/water (9/1/4, v/v/v) Bidwell, 1957) to examine for the presence of any sugars. Standard sugars were also chromatographed in the same solvent under the same conditions. The $R_{glucose}$ values of standard sugars are shown in Table XII. Sugars were detected with either ammoniacal silver nitrate or alkaline permanganate reagents. The ammoniacal silver nitrate reaction was carried out by spraying the chromatogram with a mixture of equal volumes of 0.1 N $AgNO_3$ and 5N NH_4OH followed by heating the paper under infra red lamps for 5-10 minutes when reducing sugars appear as brown spots. The alkaline permanganate reaction was carried out as in Block, Durram and Zweig (1958).

Table XII

$R_{glucose}$ values of standard sugars chromatographed in butanol, acetic acid, water (9:1:4)

Sugar	$R_{glucose}$ value
Glucose	1.0
Arabinose	1.3
Ribose	1.63
Fucose	1.78
Mannitol	1.15
Mannose	1.22
Fructose	1.15
Sedoheptulose	1.3

Results

Both the alkaline hydrolysate and the acid hydrolysate showed the presence of only one sugar spot which had an $R_{glucose}$ value of 1.14. This same region gave a positive test with the colour reagents after chromatography of untreated extract.

Chromatography of the hydrolysates in Bandurski and Axelrod's acidic solvent, followed by autoradiography showed that the only detectable radioactive region approximated to orthophosphate.

Discussion

From examination of the $R_{glucose}$ values presented it would appear that the sugar detected after hydrolysis of untreated extract, with either acid or base, could be either fructose or mannitol. Under the conditions used in hydrolysis, sugar phosphates such as glucose-1-phosphate, glucose-6-phosphate, fructose-6-phosphate, mannitol-1-phosphate, which might be expected to be present in the extract would be broken down to the corresponding sugar and inorganic phosphate. Any laminarin or fucoidin which might also be present in the extract would also have been hydrolysed, thus glucose and possibly fucose could have been expected to be present in the hydrolysates. However, as stated, the only sugar detected appears to correspond to either mannitol or fructose. Since this same region ($R_{glucose}$ = 1.14) is present in the non-hydrolysed extract, and since there is evidence that mannitol is the only free sugar found in L. digitata (Bidwell, 1958), it could be that the main bulk of the sugar found in the hydrolysates is accounted for by the original free mannitol. But this does not rule out the possibility of extra mannitol having been formed by hydrolysis of sugar esters in the extract since the colour reactions for the detection of the sugars are not quantitative. If mannitol were formed by hydrolysis

of an ester in the extract the most probable ester would be mannitol-1-phosphate. The results in Table VI showing the R_f values of standard phosphate esters and the R_f values of radioactive substances present in an alcoholic extract of L. digitata do not indicate that mannitol-1-phosphate is present. Thus a possible explanation of the results from hydrolysis of the crude extract is that mannitol shown to be present in the hydrolysates is present from the start as free mannitol. That the phosphates present in the extract are in fact hydrolysed, resulting in inorganic phosphate is shown by chromatography of the hydrolysates in the acidic solvent of Bandurski and Axelrod where no other radioactive substance other than orthophosphate was detected.

Hydrolysis of separated components of 60% aqueous ethanol extractions of L.digitata

Crude 60% ethyl alcohol extracts of P^{32} containing L. digitata were separated into the various components by chromatography in the acidic solvent of Bandurski and Axelrod. The radioactive components were detected by autoradiography and eluted from the paper with distilled water. The eluates were collected and reconcentrated under reduced pressure. Hydrolysis of the main radioactive components, including those thought to be orthophosphate, pyrophosphate and glucose-1-phosphate, was then carried out for 30 minutes at 100°C in N.HCl. Chromatography of the hydrolysates and the non-hydrolysed component was then carried out once more in the acidic solvent.

Results

Hydrolysis and chromatography of the component thought to be orthophosphate resulted in detection as expected of only orthophosphate. Hydrolysis of the substance believed to be pyrophosphate resulted in

the detection of two radioactive regions, the main one being orthophosphate, the second region corresponding to the original substance.

After hydrolysis of the substance thought to be glucose-1-phosphate and chromatography in the acidic solvent of Bandurski and Axelrod for the detection of phosphates and also chromatography in the butanol, acetic acid, water solvent for the separation of sugars, pyrophosphate and orthophosphate were detected, but no positive sugar reaction was obtained.

Discussion

From the evidence in this section and the other evidence presented on the number and nature of the phosphate compounds present in the alcoholic extract, it would appear that the substances thought to be orthophosphate and pyrophosphate are in fact those compounds. The results from the hydrolysis of the substance thought to be glucose-1-phosphate do not assist in the positive identification in the absence of a positive test for glucose.

Discussion on the number and nature of phosphorylated compounds present in a 60% aqueous alcohol extract of L. digitata

From examination of the 60% alcohol extract in the various solvent systems used, it is evident that the most useful solvent is the Bandurski and Axelrod acidic solvent. The reason for this choice is that a good, clean separation of the P^{32} labelled components is achieved in a reasonably short time.

The results of chromatography show that there are six unidentified P^{32}-labelled regions present on a chromatogram developed in Bandurski and Axelrod's acidic solvent under the stated conditions having the following R_f values:

0.18 - 0.25
0.28 - 0.34
0.41 - 0.45
0.49 - 0.55
0.57 - 0.61
0.77 - 0.82

The spread of the R_f values might be due to differences in the temperature at the time of the various runs since chromatography was carried out at "room temperature".

From the results of two dimensional chromatography in the Bandurski and Axelrod acidic and basic solvent systems (Fig. vi) it would appear that the substance R_f 0.77 - 0.81 is orthophosphate; the region R_f = 0.57 - 0.61 might be pyro- and/or metaphosphate and the region R_f = 0.49 - 0.52 could be glucose-1-phosphate. Other chromatographic techniques were employed to verify these findings. Using Ebel's acidic solvent it was possible to separate a crude extract into regions which had four main peaks of activity. From the graph of R_f value (in Ebel's solvent) v the chain length of polyphosphates it was possible to show that the fastest moving peak

activity, R_f = 0.70, corresponded to orthophosphate.

On elution of this substance and rechromatography in the Bandurski and Axelrod acidic solvent it was seen that this peak corresponded to the region having R_f = 0.77 - 0.81 in the Bandurski and Axelrod acidic solvent and no other radioactive regions were seen on the autoradiograph (Table IX). This evidence, together with the fact that P^{32} used as a standard runs to the region R_f = 0.77 - 0.81 in the Bandurski and Axelrod acidic solvent, would seem to confirm the fact that the region R_f = 0.77 - 0.81 is in fact orthophosphate.

The substance having R_f = 0.28 in Ebel's acidic solvent can be seen from the graph of log R_f v chain length of polyphosphates (Fig. viii) to approximate to pyrophosphate. After elution of the radioactivity present in this region and rechromatography in the acidic solvent of Bandurski and Axelrod, three radioactive regions were observed (Table X). From correlation with the crude extract chromatographed under the same conditions, it can be seen that present in this region R_f = 0.28 there is probably a mixture of orthophosphate, pyrophosphate and glucose-1-phosphate. As indicated by visual inspection the radioactivity was predominantly present in the pyrophosphate region. The presence of a mixture of orthophosphate, pyrophosphate and glucose-1-phosphate in this substance would explain why the R_f value was lower than that exhibited by pyrophosphate alone.

The radioactivity present in the region R_f = 0.13 and 0.06 in Ebel's solvent when eluted as one single region and rechromatographed in the Bandurski and Axelrod acidic solvent, shows a mixture of substances present (Table XI) and the only substance which is not also present in the region R_f = 0.28 is that having an R_f = 0.45 in the Bandurski and Axelrod solvent.

Thus the only radioactive substances which can be identified by paper chromatographic methods in an alcoholic extract of L. digitata labelled with P^{32} under the stated conditions are shown below (Table XII).

Table XII

P^{32} containing substances present in a 60% alcoholic extract of L. digitata

R_f value in Bandurski and Axelrod acid solvent	Substance
0.18-0.25	Unidentified
0.28-0.34	Unidentified
0.41-0.45	Unidentified
0.49-0.55	Glucose-1-phosphate
0.57-0.61	Pyrophosphate
0.77-0.82	Orthophosphate

(ii) Examination by Ion Exchange Chromatography

The strong base anion exchange resin IRA 400 was used in formate, chloride and OH forms in attempts to separate an untreated labelled alcoholic extract of L. digitata. Columns were formed as described above and liquid fractions containing peak radioactivity concentrated and chromatographed in the Bandurski and Axelrod acidic solvent.

Results from this type of investigation were not very encouraging. If extract was applied to any of the above forms of resin and the column simply washed with distilled water, there was a large peak of activity contained in the 20th to 40th ml of eluate. This peak contained the majority of radioactivity placed on the column and the only substance not also found in untreated crude extract was orthophosphate. It was thought that the reason for non-retention of the phosphorylated compounds by the

ion exchange resin might have been due to "overloading". To test this a column 20 cm x 4 cm was prepared from IRA 400 in the chloride form with again the same results as above.

The question as to why P^{32} containing substances were not retained by the column remains as yet unresolved. It might be that the presence of alginate in the extract interferes with retention of the phosphates. The precipitation of alginate then might be expected to improve matters but since the precipitation technique also precipitates phosphates as calcium phosphates this does not help to clarify the situation. For this reason and since chromatography in the Bandurski and Axelrod acid solvent gives a good separation of algal extract in a short time, ion exchange resin chromatography was abandoned.

(iii) Examination by Paper Electrophoresis

It was thought that electrophoresis might offer a quick, convenient method for the separation of P^{32} containing compounds present in a crude labelled extract of L. digitata. There is no evidence in the literature for this technique having been used for routine analysis of this type.

Investigations were undertaken to establish the electrophoretic pattern formed by the P^{32} labelled compounds present in an untreated alcoholic extract from L. digitata.

Various combinations of voltage, time and pH were examined.

Among the combinations of voltage and time investigated were those shown below:

Autoradiograph to Show the P^{32} Containing Regions Observed on Electrophoresis of L. digitata Extract at pH 4.0

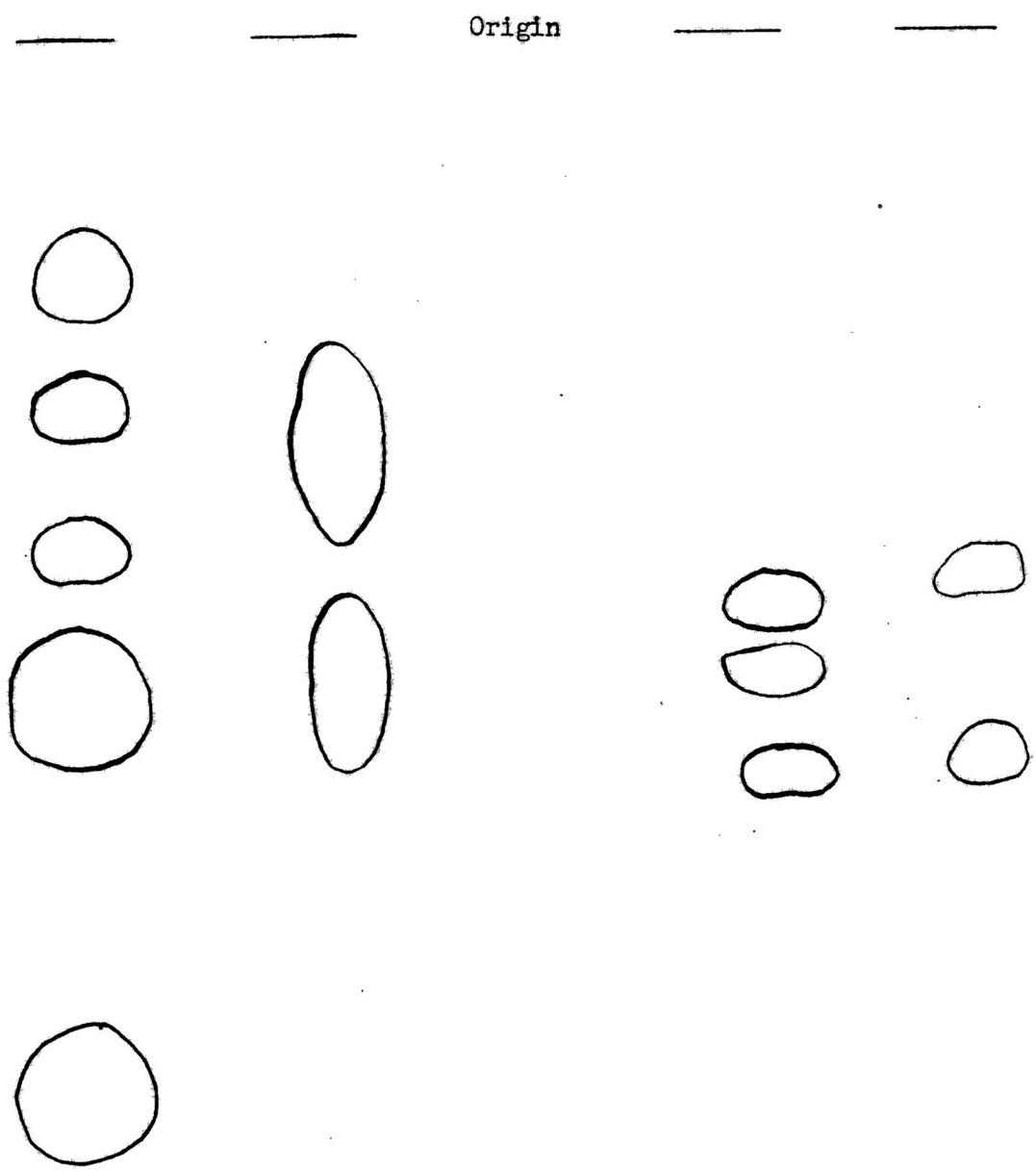
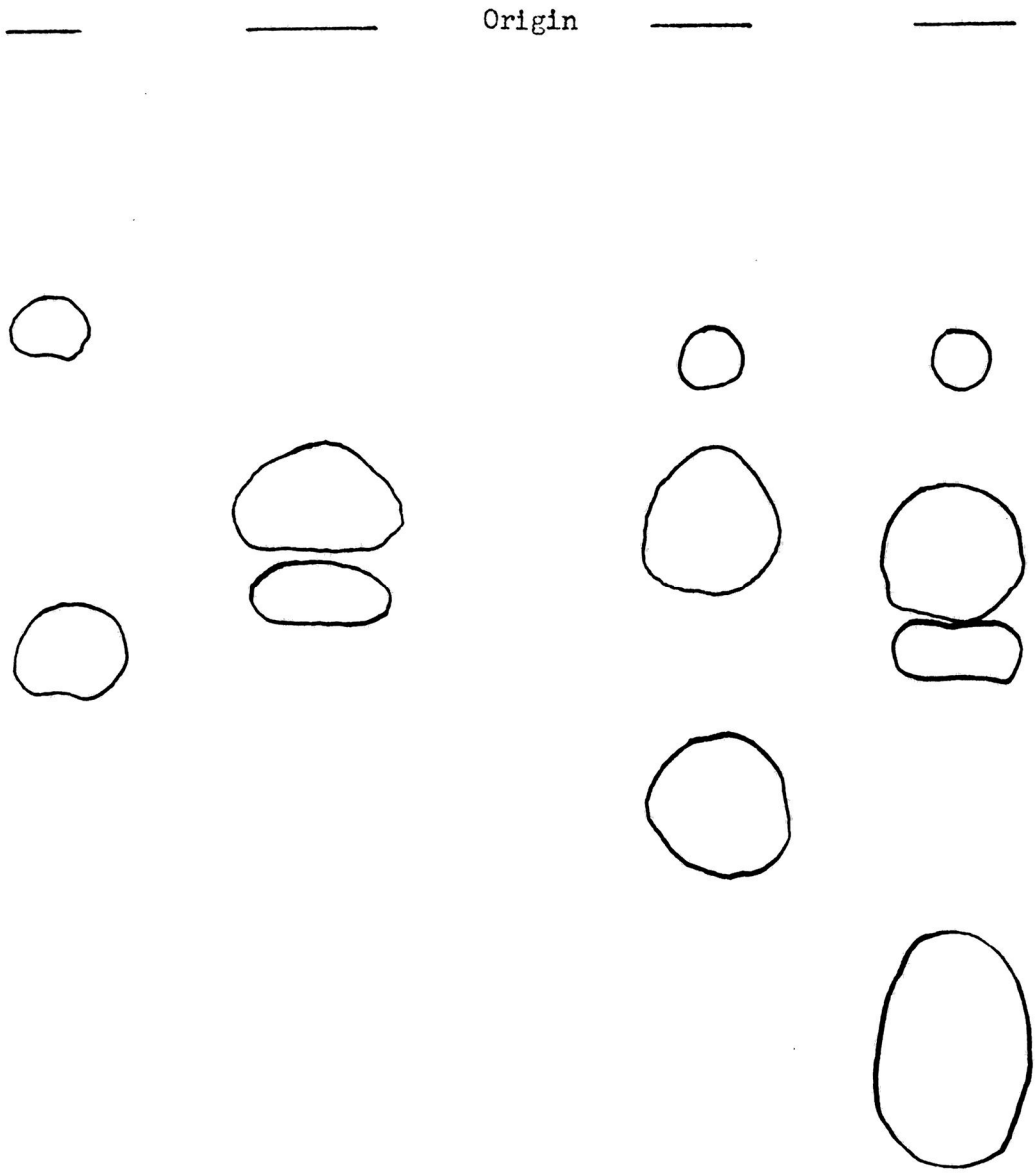


Fig. ix

Autoradiograph to Show the P^{32} Containing Regions Observed on Electrophoresis of L. digitata Extract at pH 4.0



240 v for 180 min. 300 v for 120 min.

Positive Electrode

Fig. x

volts	time	current start	current finish
300	60 min.	10 mA	20 mA
240	180 min.	15 mA	25 mA
300	120 min.	15 mA	20 mA
300	90 min.	10 mA	25 mA

Electrophoresis was carried out on Whatman 3 mm paper in a Shandon electrophoretic tank equipped with a cooling water jacket. The electrodes were connected to a voltage-regulated power pack. Typical results of electrophoresis described above are shown in copies of autoradiographs, Fig. ix and Fig. x.

The main feature of these results is the inconsistency of the electrophoretic pattern obtained. This inconsistency probably arose from local evaporation and diffusion. Because of the non-reproducibility of results, paper electrophoresis was abandoned in favour of paper chromatography.

(d) The Influence of External Conditions on the Distribution of P^{32} in the Phosphorylated Compounds Present in a 60% Aqueous Alcohol Extract from L. digitata

In this section of the work, dark adapted tissue was employed. In general, the technique was to add phosphorus^{32} to algal discs in sea water, at the same time providing illumination if required, and then to remove samples for analysis at various time intervals. The samples were quickly washed with inactive sea water and immersed in a known weighed volume of hot 60% ethanol. After weighing the ethanol plus tissue, extraction by shaking was carried out for 120 minutes. Extracts were then filtered and concentrated. Chromatography of the concentrated extracts was carried out in the acidic solvent of Bandurski and Axelrod for

six hours in the ascending direction at room temperature. Autoradiographs were prepared after chromatography and the radioactivity present in the various phosphorylated components assayed.

As well as the relative labelling of the extractable compounds present, the total uptake of P^{32} by the tissue can be obtained in the following way:

Since a known weight of tissue was extracted with a known volume of 60% ethanol, the extractable count per minute per gram fresh weight of tissue can be calculated. A sample of solvent was counted in a liquid counter and the extractable radioactivity obtained from

$$\frac{\text{cpm/ml solvent x total volume solvent in mls}}{\text{fresh weight of tissue in gms}} \qquad (1)$$

The unextractable radioactivity was measured by wet ashing a known weight of previously extracted tissue and assaying the solution in a liquid counter. The unextractable count per minute per gram fresh weight of algae is calculated from

$$\frac{\text{cpm/ml wet ashed sample x total volume of solution from wet ashed sample}}{\text{fresh weight of tissue wet ashed in gms}} \qquad (2)$$

The total uptake of phosphorus^{32} per gram fresh weight L. digitata is obtained from the summation of equations (1) and (2).

(i) Total Uptake of Phosphorus^{32}

(a) Effect of light under aerobic conditions

Dark adapted tissue was provided with phosphorus^{32} and illumination at the same instant and samples removed after 2, 5, 10, 30 and 60 mins. The samples were treated as described above. Table XIV shows the results obtained under these conditions.

Table XIV

Uptake of P^{32} by dark adapted tissue provided with light under aerobic conditions

Time	2 min.	5 min.	10 min.	30 min.	60 min.
Extractable cpm/gm fresh weight	30519	31280	32556	31321	38686
Unextractable cpm/gm fresh weight	30769	43571	48276	71200	85600
Total cpm/gm fresh weight	61288	74851	80832	102521	124286
% activity extracted	49.8	41.79	40.28	30.55	31.13

(b) Effect of darkness under aerobic conditions

Dark adapted tissue under normal aeration was provided with P^{32} and samples removed for analysis after 2, 5, 10, 30 and 60 minutes. Table XV shows the results obtained.

Table XV

Dark uptake of P^{32} by dark adapted tissue under aerobic conditions

Time	2 min.	5 min.	10 min.	30 min.	60 min.
Extractable cpm/gm fresh weight	57100	44800	63250	65900	57700
Unextractable cpm/gm fresh weight	42051	65714	73684	88148	98571
Total cpm/gm fresh weight	99151	110514	136934	154043	156271
% activity extracted	57.59	40.54	46.19	42.78	36.92

Total Uptake of P^{32} by L. digitata under the Influence of Various External Conditions

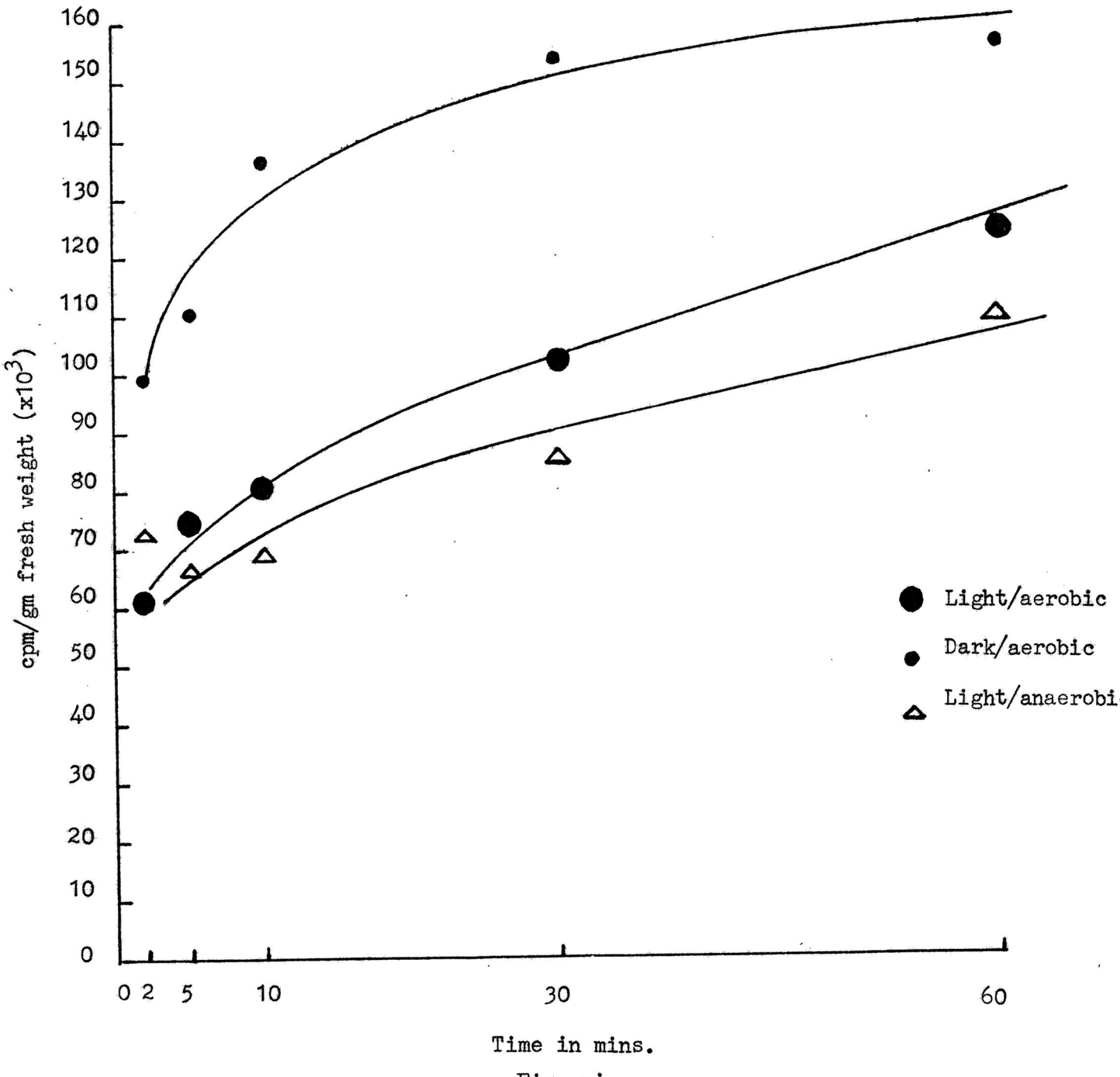


Fig. xi

(c) Effect of light under anaerobic conditions

L. digitata (dark adapted) discs under a nitrogen atmosphere were provided with P^{32} and illumination at the same instant. Samples were removed and analysed after 2, 5, 10, 30 and 60 mins. The results are presented in Table XVI.

Table XVI

Light uptake of P^{32} by dark adapted tissue under nitrogen atmosphere

Time	2 min.	5 min.	10 min.	30 min.	60 min.
Extractable cpm/gm fresh weight	32974	32742	29456	33333	33123
Unextractable cpm/gm fresh weight	40769	35172	40939	54482	73333
Total cpm/gm fresh weight	73743	67914	70395	87815	111456
% activity extracted	44.71	48.84	41.84	37.96	34.21

The above results on the effects of various external factors on the total uptake of P^{32} by Laminaria digitata are shown graphically in Fig. xi. It should be noted that Fig. xi is not intended to show the relative amounts of radioactivity incorporated into the tissue, but only the rate at which the P^{32} was taken up.

(ii) Relative Labelling of the Phosphorus^{32} Compounds Present in a 60% Aqueous Alcohol Extract of L. digitata

The relative labelling of phosphorylated compounds present in a 60% aqueous ethanol extract from L. digitata, provided with P^{32} under a variety of external conditions, was estimated by cutting out P^{32} labelled regions from chromatograms, digesting the paper in perchloric acid and measuring the radioactivity in a liquid counter.

The Effect of Light under Aerobic Conditions on the Distribution of P^{32} in the Phosphorylated Compounds Present in a 60% Aqueous Alcohol Extract of L. digitata

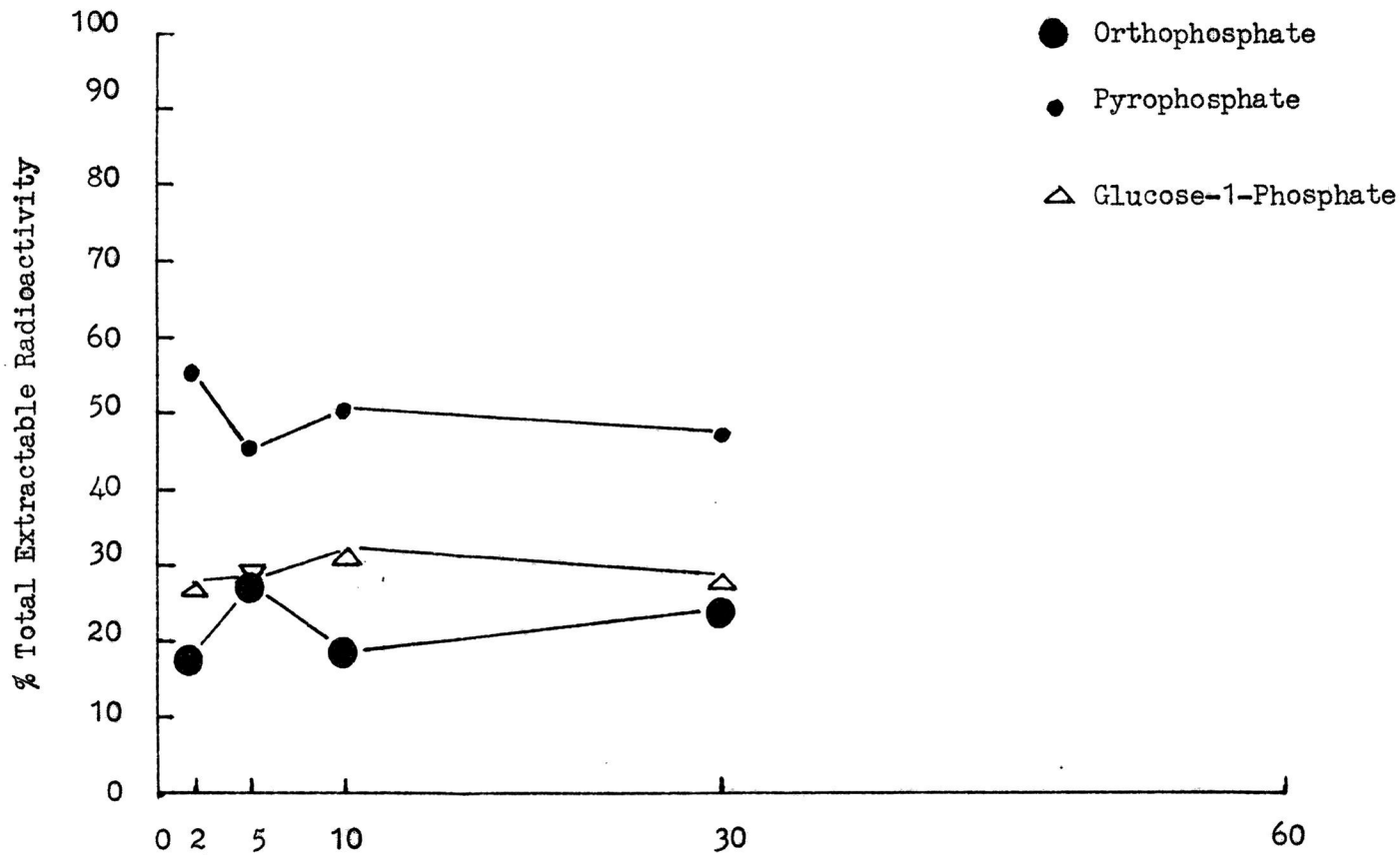


Fig. xii

The Effect of Darkness under Aerobic Conditions on the Distribution of P^{32} in the Phosphorylated Compounds Present in a 60% Aqueous Alcohol Extract of L. digitata

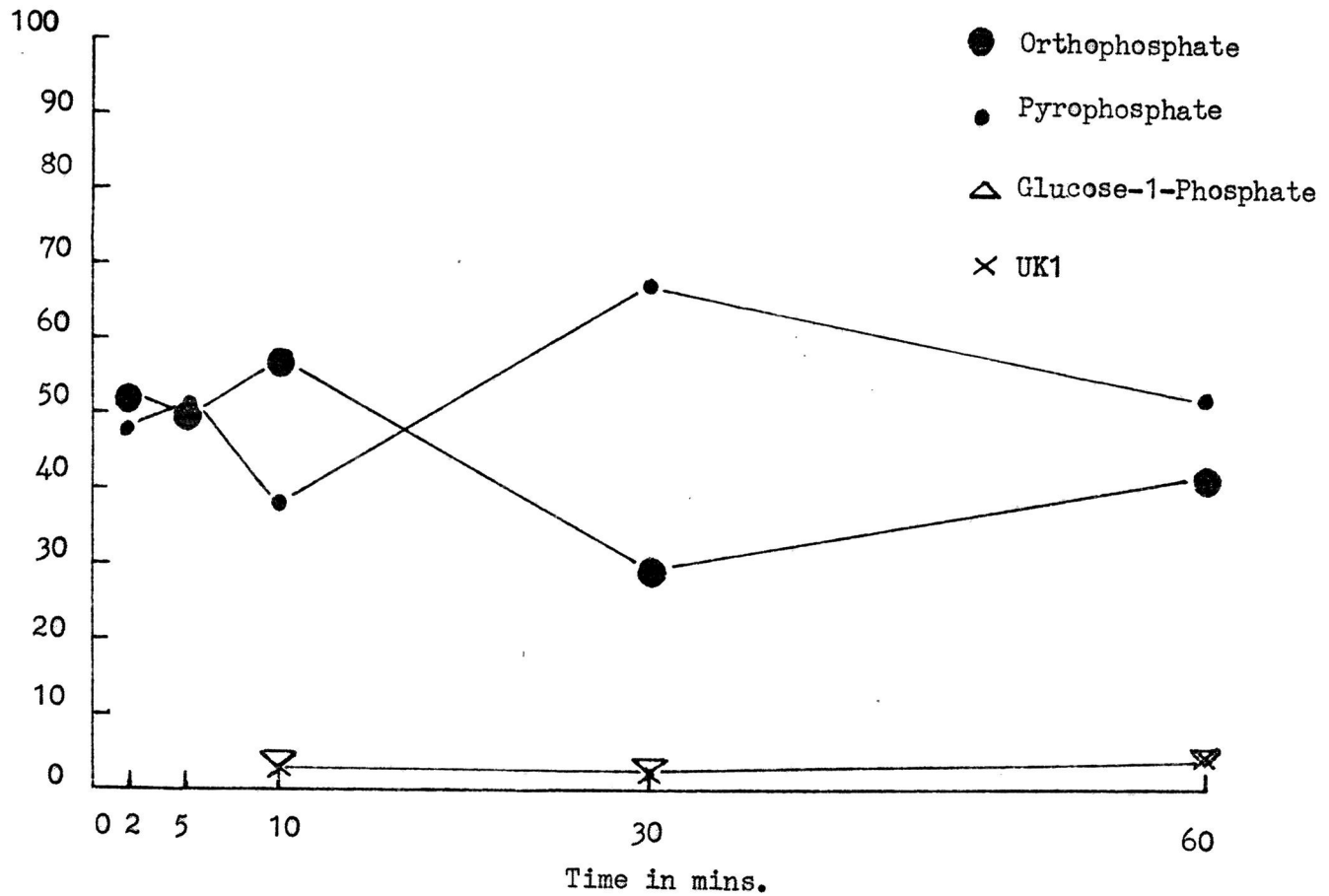


Fig. xiii

(a) Effect of light under aerobic conditions

Concentrated extracts were prepared at various time intervals from dark adapted tissue which had been supplied with P^{32} and light at the same instant under aerobic conditions. The relative labelling in P^{32} containing components, separated from the extract by chromatography in the acidic solvent of Bandurski and Axelrod is shown in Table XVII and graphically in Fig. xii.

Table XVII

Distribution of P^{32} in phosphorylated components present in 60% alcoholic extract of dark adapted L. digitata provided with light under aerobic conditions

Time	Substance	cpm	% total cpm
2 min.	orthophosphate (PO)	86	17
	pyrophosphate (PP)	270	55
	glucose-1-phosphate (G-1-P)	139	28
5 min.	PO	164	27
	PP	274	45
	G-1-P	169	28
10 min.	PO	138	18
	PP	387	50
	G-1-P	245	32
30 min.	PO	159	24
	PP	306	47
	G-1-P	187	29

(b) The effect of darkness under aerobic conditions

Dark adapted plants were provided with P^{32} and samples removed at various intervals for assay of the distribution of P^{32} in phosphorylated compounds present in a 60% alcoholic extract. The results are shown in Table XVIII and graphically in Fig. xiii.

The Effect of Light under Anaerobic Conditions on the Distribution of P^{32} in the Phosphorylated Compounds Present in a 60% Aqueous Alcohol Extract of L. digitata

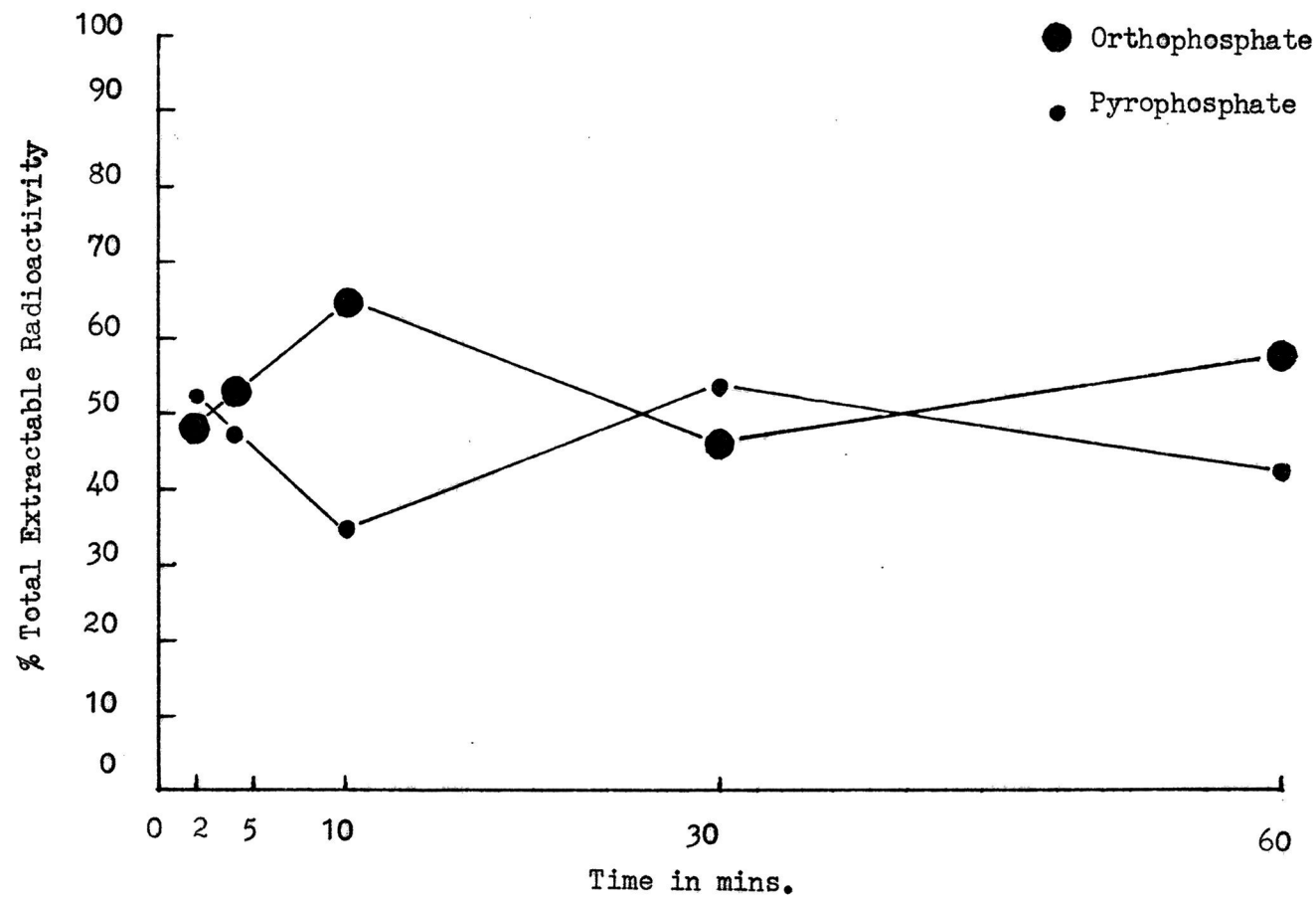


Fig. xiv

Table XVIII

Distribution of P^{32} in a 60% alcoholic extract of L. digitata provided with P^{32} in darkness under aerobic conditions

Time	Substance	cpm	% total cpm
2 min.	PO	1099	52
	PP	1024	48
5 min.	PO	731	49
	PP	753	51
10 min.	PO	397	57
	PP	263	38
	G-1-P	17	2.5
	UK1	16	2.5
30 min.	PO	692	29
	PP	1629	67
	G-1-P	55	2
	UK1	54	2
60 min.	PO	620	41
	PP	785	52
	G-1-P	47	3
	UK1	56	4

(c) The effect of light under anaerobic conditions

Dark adapted plants pretreated in the way described above were provided with phosphorus^{32} and light at the same instant. Samples were removed at various time intervals and assayed for the distribution of radioactivity in the alcoholic extract. The results are presented in Table XIX and graphically in Fig. xiv.

Table XIX

The distribution of P^{32} in phosphorylated compounds present in an alcoholic extract of L. digitata provided with P^{32} in the light under anaerobic conditions

Time	Substance	cpm	% total cpm
2 min.	PO	213	48
	PP	231	52
5 min.	PO	187	53
	PP	167	47
10 min.	PO	131	65
	PP	71	35
30 min.	PO	185	46
	PP	218	54
60 min.	PO	131	58
	PP	95	42

Discussion on the Results of Experiments to Show the Effects of External Conditions on the Total Uptake and Distribution of P^{32} in Ethyl Alcohol Soluble Phosphorylated Compounds of L. digitata

From the results presented in Fig. xi it can be seen that under the various conditions of light and atmosphere employed in the present work there was no obvious effect on the rate of uptake during the first 60 minutes of such a process.

Consideration of the results on the effect of external conditions on the distribution of P^{32} in the 60% ethyl alcohol extractable phosphorylated compounds shows that in light under aerobic conditions (Fig. xii) there is a production of glucose-1-phosphate and this accounts for approximately 30% of the total radioactivity. Under anaerobic conditions, in light, (Fig. xiv), however, there was no detectable glucose-1-phosphate detected. Since the results presented in Fig. xiii show only a very slight production of G-1-P it can be deduced that both light and air are necessary for the

formation of glucose-1-phosphate.

(e) The Sequential Formation of Phosphorylated Compounds Present in a 60% Aqueous Ethyl Alcohol Extract of L. digitata

In this section of the work the relative labelling of the various components present in an alcoholic extract of L. digitata, which had incorporated P^{32} in the light, was investigated in an attempt to determine the sequence in which they were phosphorylated under aerobic conditions. The plants used had been kept under standard storage conditions in the laboratory and P^{32} was administered in the light at room temperature. Samples of tissue were removed at various time intervals for extraction and subsequent assay of radioactivity in the P^{32} labelled components present in the aqueous alcoholic extract. Relative labelling of the phosphorylated compounds was determined after chromatography and autoradiography. Table XX shows the relative labelling of components after incorporation of P^{32} for 24 hours (12 hours light, 12 hours dark).

Table XX

Relative labelling of the compounds present in a 60% alcoholic extract of L. digitata after P^{32} incorporation for 24 hours

Compound	% Total Radioactivity
PO	7.3%
PP	12.7%
G-1-P	41.6%
UK1	28.7%
UK2	4.0%
UK3	5.7%

The results for the relative labelling after time intervals of up to 60 minutes are presented in Table XXI.

The Relative Labelling of P^{32} Containing Substances Present in L. digitata Which Had Incorporated P^{32} in the Light Under Aerotic Conditions.

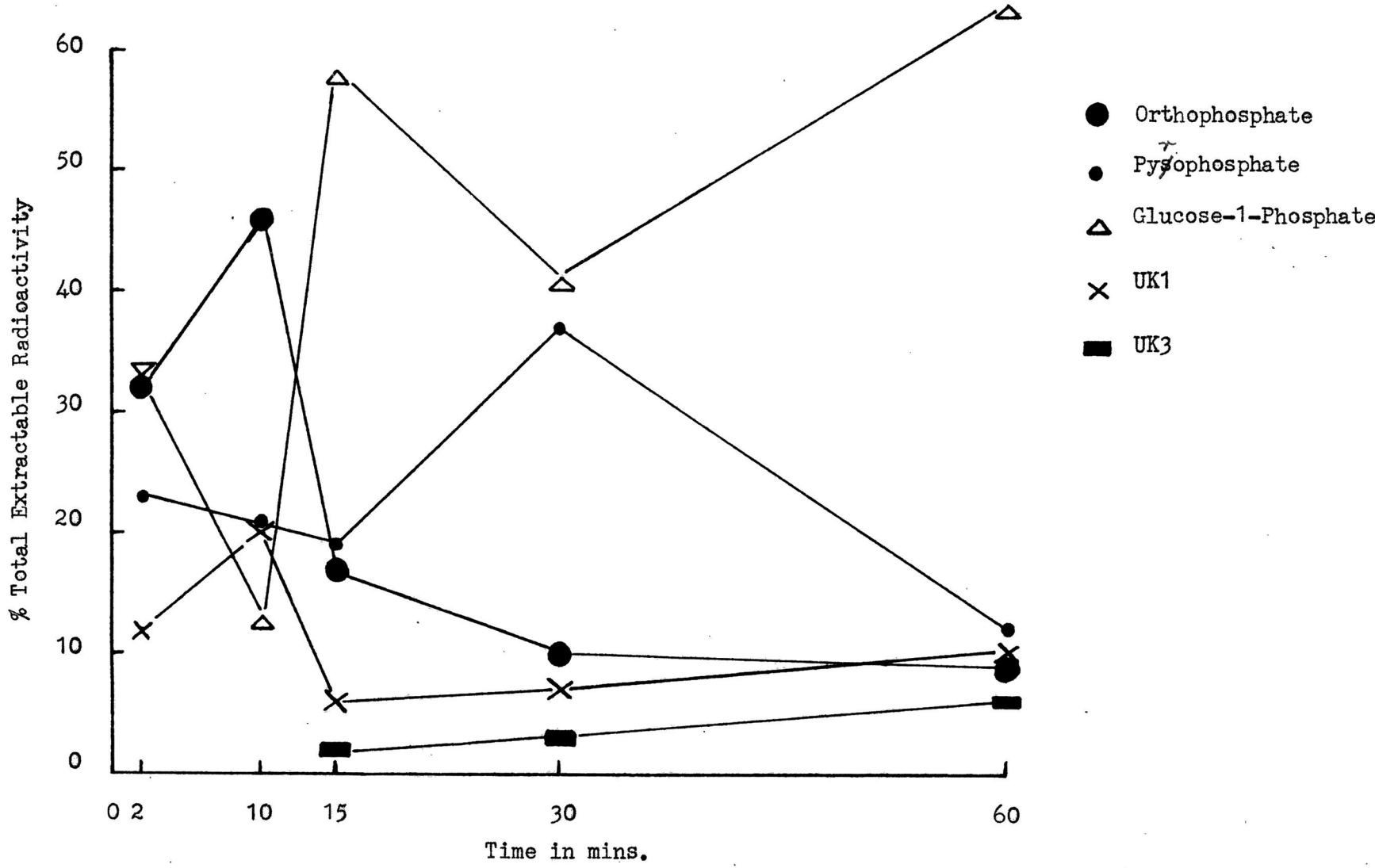


Fig. xv

Table XXI

The relative labelling of P^{32} containing substances present in L. digitata which had incorporated P^{32} in the light under aerobic conditions

Substance	Time				
	2 min.	10 min.	15 min.	30 min.	60 min.
PO	32%	46%	17%	10%	9%
PP	23%	21%	19%	37%	12%
G-1-P	33%	13%	58%	41%	64%
UK1	12%	20%	6%	7%	10%
UK2	not detected	not detected	not detected	not detected	not detected
UK3	not detected	not detected	2%	3%	6%

The above results are present graphically in Fig. xv.

Discussion

By feeding a system with a labelled substrate and taking samples for analysis at time intervals thereafter, it is often possible to follow the pathway by which the substrate is metabolised. In the present work radioactive phosphorus was fed into L. digitata discs and the radioactivity present in the alcohol extractable substances assayed. The percentage activity present in each substance was plotted against time. In an ideal situation, provided a sufficiently short incubation time is taken, only the first product of metabolism will be labelled. Thus 100% of the activity will be found in the first product of metabolism. Thereafter the activity in this substance declines and that in the second rises. This reaches a peak and declines in turn as the third product is formed. In this way the sequence of formation of compounds along the metabolic chain can be found. However, in practice, a number of difficulties arise. A major difficulty is that of obtaining a sufficiently brief incubation time to find all the radioactivity in one substance. In the present work with L. digitata the shortest practicable incubation time was two minutes, after

which radioactivity was found in four of the six aqueous alcohol extractable compounds. A further difficulty is encountered in metabolic pathways which branch or when the substrate is taken up at more than one point. Cyclic pathways introduce more difficulties. It should also be noted that a sequence of peak formations with time, e.g. peak A followed by peak B followed by peak C, does not necessarily mean that substance B is formed from substance A, or that substance C is formed from substance B since this sort of situation may arise from parallel reactions being carried out at different rates. In the present study in the metabolism of radioactive phosphorus in Laminaria digitata we have a very complex system to analyse and the picture presented by analysis of the type described above shows the overall situation. A simple examination of the peaks shown in Fig. xv would indicate that orthophosphate, pyrophosphate, glucose-1-phosphate and UK1 are all labelled at the end of a two minute incubation with P^{32}. After this PP and UK1 continue to increase their percentage of radioactivity at the expense of G-1-P and PO, the most striking reduction being that of G-1-P. In the time interval, 10-15 minutes of incubation with P^{32}, there is a rapid decline in the radioactivity present in the inorganic phosphate fraction (PO) and in the UK1 fraction with a much slower fall in the activity present in PP. There is a most marked rise in the level of P^{32} in G-1-P during this same time. From 15 minutes to 30 minutes incubation with radioactive phosphate shows the largest gain in activity of the PO fraction with a fall in the G-1-P activity which is still the dominant P^{32} containing substance. At the 15 minute sample UK1 and UK3 are first detected, which two compounds continue to gain radiophosphate slowly until, as shown in Table XIX, after 24 hours labelling UK1 contains 28.7% of the total activity, UK2 contains

only 5.7%. Examination of the results shows that in the period 30-60 mins. there is an overall rise in the G-1-P activity with a decline in the PP fraction.

Interpretation of the results is made difficult by not having knowledge of the site of origin of the compounds present. We assume that the PO fraction is intracellular in nature. The origin of G-1-P could be the cell surface or intracellular, as with the PP. Another problem which presents itself in an attempt to elucidate the sequence in which the phosphorus containing compounds are phosphorylated, is the nature of the process whereby externally placed inorganic phosphate enters into the intracellular phosphate cycle. Two types of mechanism have been proposed. One mechanism is based on the idea that exogenous phosphate enters as inorganic orthophosphate by diffusion and mixes with intracellular orthophosphate. This intracellular phosphate is assumed to be the source from which the various organic phosphates in the cell derive phosphate. Another mechanism supposes the entry of phosphate involves esterification at the cellular interface and the production of intracellular orthophosphate from breakdown of the esterified form. Evidence from the effect of inhibitors and illumination on the P^{32} uptake process would appear to favour the esterification mechanism as the primary cause of P^{32} incorporation.

In the light of this, the present results could be interpreted as the esterification of G-1-P or UK1 at the cell interface. From consideration of the rate of labelling of UK1 and G-1-P as shown by Fig. xv, it would seem more likely that G-1-P was esterified first. After this initial stage, hydrolysis of G-1-P could explain the build up of PO and increase in UK1.

Another possible interpretation of the results for the short time labelling experiments is that the early, rapid, rise in activity may not be caused metabolically but are due to radioactive medium penetrating into the free space of the tissue (the definition of the term "free space" used here is that of Briggs & Robertson, 1957). Subsequent extraction techniques may cause the release of organic forms of phosphate from the inside of cells on to which the carrier-free phosphate can then readily absorb. However, if the results from experiments on the incorporation of radioactive phosphorus under various external conditions are taken into consideration it can be seen that in darkness under anaerobic conditions only PO and PP are labelled. If the "free space" theory was in fact correct, we would expect, even under these conditions, to find that G-1-P and UK1 contained P^{32}.

In summary, then, the changes in concentrations of radioactive label of the various intermediates found from analysis of the sequential formation of these intermediates are in fact truly metabolic. Up to 2 minutes incubation with P^{32} results mainly in the phosphorylation of G-1-P with PP and UK1 being phosphorylated at a slower rate, perhaps after hydrolysis of G-1-P inside the cell releasing PO or even PP itself. In the 2 minute - 10 minute period the main change is a fall in radioactivity associated with G-1-P and an increase in the PO fraction. After 10 minutes the PO fraction falls to a steady level and the chief P^{32} containing compound becomes G-1-P.

GENERAL DISCUSSION

The present work was undertaken to gain some knowledge of the factors affecting the uptake of phosphorus by marine algae and to investigate the subsequent phosphate metabolism after entry into the algal cells.

When dealing with phosphate metabolism in any cell it is evident that the phosphorylated sugars are going to be prominent components of the reacting system. These sugar phosphates are likely to be present in extremely low concentrations and are of a very transient nature. Consequently, the analysis of the particular sugar phosphates present in a tissue at a chosen time is an extremely difficult task. The use of chromatography and radioactive tracers helps to overcome these difficulties by virtue of the high sensitivity of the methods.

In the present work an attempt was made to discover factors affecting the uptake and metabolism of radioactive phosphorus by the marine alga Laminaria digitata. The major difficulty in the course of the present work lay in the identification of P^{32} containing substances extracted from L. digitata by 60% aqueous alcohol. Scott & Thomson (1959) who showed that the majority of P^{32} taken up by L. digitata could be extracted by 60% aqueous alcohol, suggested that either glucose-6-phosphate or possibly mannose-6-phosphate were present in a similar extract. In the present work the only compounds identified with any certainty are orthophosphate, pyrophosphate and glucose-1-phosphate.

It was obvious that for analysis of this type large quantities of substances were required and it was thought that the use of an ion exchange resin would provide a quick convenient method of separating the sugar phosphates present in the plant extract. However, it was found that there

was no retention of the sugar phosphates by the column, the only substance remaining on the column being orthophosphate. As reported previously, the reason for this curious result is as yet unresolved. Electrophoresis was another technique employed in an effort to get quick separation of extract with a view to bulk collection of sugar phosphates for analysis, but again this technique did not yield any results of value from this aspect. Thus the majority of the work has been carried out employing chromatography as the main technique for separation of the plant extract into readily recognizable fractions.

There appears to be some doubt in the literature as to the origin of inorganic phosphate obtained from TCA extractions of the plant material. The question arises as to whether the inorganic phosphate is truly intracellular inorganic phosphate or whether the inorganic phosphate results from hydrolysis of existing organic phosphate by the acid during extraction. For this reason a comparison of TCA extracts and aqueous ethyl alcohol extracts was made to determine the extracting solvent which would minnimise any error which might be present in the quantitative analysis of phosphate compounds extracted. As reported, there is no difference qualitatively in the nature of the P^{32} compounds extracted by TCA and by aqueous ethyl alcohol but there is a difference in the relative proportions of the various phosphorylated compounds. In fact TCA would appear to extract more orthophosphate than does ethyl alcohol (Table V). It is of interest to note here that extraction with a variety of solvents ranging from boiling water to cold water and with various concentrations of aqueous alcohol all resulted in extraction of the same phosphorylated compounds. That these phosphates were truly extracted from the plant and not merely artifacts of the method of concentrating the extracts was shown by the fact

that no other phosphates are formed by concentration than are present in the plant extract, nor are any phosphates lost on concentration. Furthermore, the method of concentration, whether by freeze drying, evaporation under reduced pressure, either aerobically or anaerobically, did not change the chromatographic picture.

The problem of elucidation of the metabolism of phosphorus can be approached from two different paths. Isolated enzyme systems can be studied and the whole linked together, or the plant can be studied as a whole unit; the latter was the approach adopted in the present work. Obviously, the method of investigation involved the unravelling of an extremely complex system as phosphate might enter the plant by a variety of routes and also enter into a number of cyclical systems. If this is the case, the picture obtained from the present work must be the overall picture.

There are two schools of thought as regards the mode of entry of phosphate into plant tissue. One theory is that the phosphate diffuses across the membrane into the cell as inorganic phosphate. The second, most widely held theory, is that esterification takes place at the surface of the cell, the phosphate is carried across the cell as an ester and hydrolysis of this ester releases inorganic phosphate into the interior of the cell.

The effect of various factors on the uptake of phosphorus by plants has already been discussed in the present work. A study of the results reveals the following facts. There is an increase in the total amount of alcohol insoluble radiophosphorus when uptake is allowed to proceed in the light, compared with the alcohol insoluble phosphate level reached with corresponding experiments in the dark (Tables XIV, XV, XVI). This

conclusion appears to be in agreement with the results for Chlorella given by Kamen and Spiegelman (1948) who demonstrated an increased turnover of phosphate in the TCA-insoluble fraction of the plant.

The imposed conditions do not appear to affect the relative rates of uptake of phosphate over the first 60 minutes for uptake under light with normal aeration, under dark with aerobic conditions or under light with anaerobic conditions (Fig. xi).

The pattern of distribution of radioactive phosphate shows that under light with normal aeration the majority of phosphorus^{32} (approx. 50%) is found in pyrophosphate. Approximately 30% of the activity is in glucose-1-phosphate and the remaining 20% in orthophosphate (Table XVII). Under the remaining combinations of conditions used, i.e. light with anaerobic atmosphere and dark, aerobic conditions, the radiophosphate for the most part was distributed between the inorganic phosphates. From this it is possible to deduce that both light and an aerobic atmosphere are necessary for the formation of G-1-P. Or, on the other hand, it is possible, but less likely, that under conditions of light and oxygen the glucose-1-phosphate formed in the light is not used up as quickly as under conditions which have sufficient illumination to produce glucose-1-phosphate but little oxygen. The sequential formation of the phosphates found in the present work has been discussed in some detail earlier.

The products of photosynthesis of brown marine algae were shown to be quite different from those of land plants (Bidwell, 1958). Bidwell demonstrated that mannitol was the major labelled product in L. digitata by the use of carbon 14. The route of formation of the hexitol is not yet known and it is tempting to speculate on the formation of mannitol by

the enzymatic reduction of glucose via the phosphorylated sugars, e.g.

$$\text{G-1-P} \rightleftharpoons \text{G-6-P} \rightleftharpoons \text{M-1-P} \rightleftharpoons \text{Mannitol}$$

Another scheme for the possible route of formation of mannitol was put forward by the Japanese group of Yamade *et al.* (1961) working with the fungus *Piricularia oryzae*. This fungus accumulates large amounts of mannitol and the results obtained by this group proved that in *P. oryzae* mannitol was produced from F-6-P by first of all F-6-P being reduced enzymatically to M-1-P in the presence of DPNH ($NADH_2$) and the resulting mannitol phosphate being hydrolysed to mannitol by a specific phosphatase. The elucidation of the photosynthetic route for the formation of mannitol in *Laminaria digitata* and the possible role of phosphorylated intermediates are two topics which require further investigation.

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SUMMARY

A study was made of the aqueous ethyl alcohol-soluble, phosphorus containing compounds present in the marine alga Laminaria digitata. The objects of the investigation were (i) the identification of the phosphorus containing substances present in an aqueous alcoholic extract of Laminaria digitata; (ii) the investigation of the effect of external conditions on the uptake of radioactive phosphorus and subsequent distribution within the alcohol soluble compounds; (iii) the determination of the sequential labelling of the alcohol soluble phosphates extracted from the tissue.

Laminaria digitata which had incorporated radioactive phosphorus was extracted with 60% aqueous ethanol and the extract concentrated. The phosphorus containing substances present in the extract were compared chromatographically with those obtained after extraction of similar material with cold trichloracetic acid and the chromatographic patterns shown to be identical. Concentrated aqueous alcoholic extracts were subjected to analysis for the identification of phosphorus containing substances. Separation of the extract was attempted by means of ion-exchange chromatography, paper electrophoresis and paper chromatography. Since the results obtained by paper electrophoretic methods were inconsistent, and those obtained from attempted ion exchange chromatography were of little value in the problem of the separation of the extract into its components, paper chromatography, which gave a good, quick separation of extract, was the most extensively used analytical technique. Six discrete radioactive substances were demonstrated on chromatography of the extract and of these six, three remained unknown and the others were shown to be orthophosphate,

pyrophosphate and glucose-1-phosphate.

The effects were investigated of various combinations of external conditions of light, darkness and atmosphere on the rate of uptake and subsequent distribution of radioactive phosphorus within the labelled compounds present in the concentrated aqueous alcoholic extracts of the tissue. The results presented suggest that in Laminaria digitata both light and air are necessary for the labelling of glucose-1-phosphate by radioactive phosphorus. Under light with anaerobic conditions, or darkness with aerobic conditions, only the orthophosphate and pyrophosphate fractions of the extract were shown to become radioactive.

Labelling of Laminaria digitata sections by radioactive phosphorus has been carried out for periods ranging from a few minutes to twenty-four hours in an attempt to deduce the sequential formation of the phosphorus containing components present in an aqueous alcohol extract. From these experiments it is concluded that the primary labelled compound which is extractable is glucose-1-phosphate.

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